Attorney Docket No.: 6001.204-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Box PCT
Commissioner for Patents
Washington, DC 20231

Sir:

This is a request for filing a patent application under 35 U.S.C. 371 of the international application identified below:

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DK00/00460	**August 21, 2000**	**September 1, 1999**

APPLICANT(S)/INVENTOR(S)		
First Name	Middle Name	Family Name
1.Sven 2.Hanne 3.	1. 2.Vang 3.	1.Pedersen 2.Hendriksen 3.

TITLE OF THE INVENTION

Maltogenic Amylase-Modified Starch Derivatives

1. This is a first submission of items concerning a filing under 35 U.S.C. 371.
2. This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
3. The US has been elected by the expiration of 19 months from the priority date (Article 31).
4. A copy of the International Application as filed (35 U.S.C. 371(c)(2)) has been communicated by the International Bureau.
5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) have not been made and will not be made.
6. An application data sheet is enclosed.
7. Priority of Danish application no. PA 1999 01219 filed on September 1, 1999 is claimed under 35 U.S.C. 119(a)-(d). A certified copy was filed in PCT/DK00/00460.
8. The benefit of U.S. provisional application no. 60/151,847 filed on September 1, 1999 is claimed under 35 U.S.C. 119(e).
9. An oath or declaration of the inventors is submitted herewith.

10. An Information Disclosure Statement is submitted herewith.

11. An Assignment and Recordation Form Cover Sheet are submitted herewith.

12. A Preliminary Amendment is submitted herewith.

13. The assignee of the present application is:

Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

Applicants request that the assignee information be included in the published patent application.

14. Direct all correspondence to Customer Number 25908:

PATENT TRADEMARK OFFICE

25908

15. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$1,040.00
CLAIMS FEE	
Total Claims: 21 - 20 = 1 x 18 =	$18.00
Independent Claims: 1 - 3 = 0 x 84 =	$0.00
Total Claims Fee:	$18.00
FEE FOR RECORDING ASSIGNMENT	$40.00
TOTAL NATIONAL FEE	$1,098.00

16. Please charge the required fee, estimated to be $1,098, to Novozymes North America, Inc., Deposit Account No. 50-1701. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: February 28, 2002

Elias J. Lambiris, Reg. No. 33,728
Novozymes North America, Inc.
405 Lexington Avenue, Suite 6400
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(212) 867-0123

Attorney Docket No.: 6001.204-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Pedersen et al.

Confirmation No: To be assigned

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: February 28, 2002

Examiner: To be assigned

For: Maltogenic Amylase-Modified Starch Derivatives

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, DC 20231

Sir:

Before examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

At page 1, after the title, insert

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 35 U.S.C. 371 national application of PCT/DK00/00460 filed August 21, 2000 (the international application was published under PCT Article 21(2) in English) and claims, under 35 U.S.C. 119, priority or the benefit of Danish application no. PA 1999 01219 filed September 1, 1999 and U.S. provisional application no. 60/151,847 filed September 1, 1999, the contents of which are fully incorporated herein by reference.--

Please replace the sequence listing with the attached sequence listing at the end of the specification.

IN THE CLAIMS:

Please cancel claims 1-27 without prejudice or disclaimer. Please add new claims 28-48:

28. A method for preparing a modified starch derivative, comprising:

(a) treating a starch derivative with a maltogenic amylase to form the modified starch derivative, wherein the maltogenic amylase has an amino acid sequence of residues 1-686 of SEQ ID NO: 1 or is a variant thereof, wherein the variant

(i) has maltogenic amylase activity;

(ii) has at least 70% identity to the amino acid sequence of residues 1-686 of SEQ ID NO: 1, and

(iii) has optimum maltogenic amylase activity in the pH range 3.5-7.0; and

(b) optionally recovering the modified starch derivative.

29. The method of claim 28, further comprising chemically deriving the modified starch derivative formed in step (a).

30. The method of claim 28, wherein step (a) is carried out at a temperature above 65°C.

31. The method of claim 30, wherein step (a) is carried out at a temperature about or above 70°C.

32. The method of claim 28, wherein the modified starch derivative has a mean molecular weight in the range of from 75,000 to 750,000, when determined by Gel Permeation Chromatography.

33. The method of claim 28, wherein the starch derivative has been chemically derived in such a way that the modified starch derivative contains a hydrophobic group or both a hydrophilic group and a hydrophobic group.

34. The method of claim 33, wherein the hydrophobic group comprises at least 5 carbon atoms.

35. The method of claim 34, wherein the hydrophobic group is selected from the group consisting of fatty acid, alkyl, alkenyl, aralkyl and aralkenyl.

36. The method of claim 28, wherein the starch derivative is an octenyl succinate derivative.

37. The method of claim 28, wherein the starch derivative has been chemically derived in such a way that the modified starch derivative contains a hydrophilic group.

38. The method of claim 28, wherein the variant has a residual maltogenic amylase activity of at least 25% after incubation with 1 mM Ca++ at pH 4.3, at 80°C for 15 minutes.

39. The method of claim 28, wherein the maltogenic amylase is a variant that has at least 80% identity to the amino acid sequence of residues 1-686 of SEQ ID NO: 1.

40. The method of claim 28, wherein the variant has optimum maltogenic activity in the pH range 4-5.5.

41. The method of claim 28, wherein the maltogenic amylase has an amino acid sequence of residues 1-686 of SEQ ID NO: 1.

42. A modified starch derivative obtained by the method of claim 28.

43. An emulsion comprising the modified starch derivative of claim 42.

44. A food product comprising the emulsion of claim 43.

45. A beverage flavor concentrate comprising the emulsion of claim 43.

46. A flavouring agent comprising the emulsion of claim 43.

47. A paper product, wherein the paper product has been treated with the modified starch derivative obtained by the method of claim 37.

48. A paper product comprising the modified starch derivative obtained by the method of claim 37.

REMARKS

Claims 1-27 have been rewritten as claims 28-48 to conform to U.S. practice. There is no new matter added, and entry of the amendment is respectfully requested.

Applicants also enclose a paper copy and a computer readable form of a Sequence Listing. The content of the paper and of the computer readable form is the same. This submission contains no new matter.

The Examiner is hereby invited to contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

Date: February 28, 2002

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Attorney Docket No. 6001.204-US PATENT

IN THE UNITED STATED DESIGNATED/ELECTED OFFICE (DO/EO/US)

INTERNATIONAL APPLICATION NO.: PCT/DK00/00460

INTERNATIONAL FILING DATE: **August 21, 2000**

PRIORITY DATE: **September 1, 1999**

TITLE: Maltogenic Amylase-Modified Starch Derivatives

APPLICANT(S) FOR RO/US: Pedersen et al.

EXPRESS MAIL CERTIFICATE

Box PCT
Commissioner for Patents
Washington, DC 20231

Sir:

Express Mail Label No. EL 896275479 US

Date of Deposit: February 28, 2002

I hereby certify that the following attached papers or fee

1. Transmittal Letter to the DO/EO/US (in duplicate)
2. Executed Combined Declaration and Power of Attorney
3. Application Data Sheet
4. Preliminary Amendment
5. Recordation Form Cover Sheet
6. Assignment
7. Paper Copy of Sequence Listing
8. Disk Containing Sequence Listing
9. Information Disclosure Statement
10. Form PTO 1449
11. Copy of References

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, DC 20231.

Julie Tabarovsky
(Name of person mailing paper(s) or fee)

J. Tabarovsky
(Signature of person mailing paper(s) or fee)

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101 Rec'd PCT/PTO 28 FEB 2002
PCT/DK00/00460
10/069908

MALTOGENIC AMYLASE-MODIFIED STARCH DERIVATIVES

FIELD OF THE INVENTION

The present invention relates to methods for preparing enzymatically modified starch derivatives useful for, e.g., being incorporated in emulsions. The present invention also relates to enzymatically modified starch derivatives per se as well as use of modified starch derivatives for preparing various food products.

BACKGROUND OF THE INVENTION

A variety of chemical compositions are used as emulsifying agents in food, cosmetics, paint, pharmaceutical and polymer industries, as well as in textile and leather processing, ore flotation, oil drilling and agricultural spraying operations. In many of these applications, the emulsifying agent also functions as a stabilizer of the viscosity or fluidity of the continuous phase. Frequently, these applications require that the emulsion be shelf stable for long periods of time.

Typical compositions which function as water soluble emulsifiers and stabilizers include guar gum, gum arabic, and other gums, starches, proteins, various water-soluble polymers, and the like (see, for example, Encyclopedia of Chemical Technology, Kirk-Othmer (Editor), 3rd Edition, Wiley-Interscience, New York, N.Y., 1979, Vol. 8, pp. 900-910, 918, 923-25). Gum arabic is preferred in many applications for its shelf stability, particularly during refrigerated or frozen storage of the emulsion. Gum arabic is a branched, substituted heteropolysaccharide characterized by extreme water-solubility, low viscosity, and the absence of odour, colour or flavour. Gum arabic has been used as an emulsifier and stabilizer in foods, such as confections, syrups, flavour oil emulsions, ice cream and beverages, and in inks, adhesives, textiles and lithography solutions.

Gum arabic is a naturally occurring gum which is grown in the Middle East and Africa. Because gum arabic is obtained from these areas, it is expensive, and its supply and quality is unpredictable. Accordingly, industry has long searched for a shelf stable, low cost replacement for gum arabic, and starch derived products have been suggested for such use.

US 2,661,349 discloses substituted dicarboxylic acid anhydride starch half ester derivatives. Some of these derivatives form stable oil-in-water emulsions suitable for use in beverage emulsion, flavour emulsion, and other emulsion applications. (see, e.g., P.Trubiano, Chapter 9, in Modified Starches: Properties and Uses, CRC Press,

Boca Raton, Fla., 1986, pp. 134-47). Cold water soluble, low viscosity octenylsuccinate starch derivatives have been successfully used to replace gum arabic in carbonated beverages. Higher viscosity octenylsuccinate derivatives have been useful as gum arabic replacers in salad dressings. Such substituted dicarboxylic acid starch half ester derivatives have also been used in place of gum arabic to encapsulate hydrophobic substances, such as flavours, vitamins, fragrances and oils.

The encapsulation is typically prepared by spray-drying an oil-in-water emulsion. Some of these encapsulating agents have been modified to yield a composition which provides a gradual or controlled release of the entrapped flavour or oil. Others can be dissolved in water at higher solids than their gum arabic counterparts and may be superior to gum arabic in certain applications.

The low viscosity (converted) starches which are used in beverage and flavour emulsions are usually prepared by acid degradation of the base starch. Processes for producing low viscosity starches are well-known. US 4,035,235 discloses a method for degradation of lipophilic substituted starches, which uses alpha-amylase digestion as an alternative to acid degradation for preparing low viscosity starches. The lipophilic substituted starches are suitable for flavour encapsulation and oil-in-water emulsions. These conversion methods produce starch products, which are suitable as emulsifiers and encapsulating agents for oils used in beverages.

One drawback to the use of the known starch derived products in replacing gum arabic is that known starch derivatives are less stable during storage. These starch derivatives display shorter shelf-life and poorer refrigeration and freeze/thaw stability than gum arabic. Therefore, in certain applications, such as flavoured syrup bases used in the manufacture of soft drinks and similar types of beverages, these starch derived substitutes do not perform as well as gum arabic. Because beverage manufacturers ship flavoured syrup bases to bottlers in diverse locations where the syrup bases may be held in refrigerated storage for long periods of time prior to use in the bottling operation, the flavour oil emulsion must remain stable during storage. In addition, because refrigeration temperatures may vary from bottler to bottler, or from day to day, the flavour oil emulsion must be able to withstand temperature cycling, including freeze/thaw cycles. The stability problem in beverage applications is thought to occur because of the tendency of starch products to retrograde, causing the flavour oil emulsion to break down upon temperature cycling or long-term storage. In severe cases, the starch may retrograde to form a gel and the flavor oil may separate entirely from the water phase. Retrogradation of starches is essentially a crystallization process that occurs when linear portions of the starch molecules align themselves next

to each other and form interchain hydrogen bonds through the hydroxyl groups. When sufficient interchain bonding occurs, the molecules associate to form molecular aggregates, which display a reduced capacity for hydration and, therefore, lower water solubility. These aggregates may precipitate, or, in more concentrated solutions, may form a gel. The tendency to retrograde is more pronounced in starches containing high levels of the linear amylose molecule. In starches containing both linear (amylose) and branched (amylopectin), or only branched molecules, the tendency to retrograde is less pronounced. As the temperature is lowered, both amylose and amylopectin containing starches display a greater tendency to retrograde.

Retrogradation has been partially overcome in certain applications by chemically derivatizing the starch molecule to stabilize the starch by interfering with the association between starch molecules, or portions of the same molecule, and thereby reducing the tendency of the starch to lose its hydration ability on storage. For example, reacting the starch with a reagent to introduce substituents such as hydroxypropyl, phosphate, acetate or succinate groups tends to stabilize the starch molecule during storage.

These derivatization reactions may be carried out on starches which are further modified by cross-linking or degradation to obtain starches for particular applications. However, these derivatized starches do not provide the stable emulsification properties, which are typical for gum arabic.

Other processes known to limit starch retrogradation at low temperatures also do not provide stable emulsifying starches. For example, US 3,525,672 discloses treating a cross-linked, inhibited starch thickener with an enzyme such as beta-amylase to impart freeze/thaw stability to starch thickeners for pie fillings, puddings and other thickened foods which are subjected to low temperature storage. It is stated that in addition to the described inhibition procedure, it is sometimes advantageous to partially derivatize the starch bases. Typical substituent groups include ester groups such as acetate, succinate, phosphate and sulfate groups as well as ether groups.

US 4,428,972 discloses a waxy starch thickener with superior low temperature stability in aqueous dispersions, which starch is obtained from a selected plant of a wxsu sub 2 genotype.

US 4,977,252 discloses the preparation of a modified starch derivative suitable for being incorporated in emulsions. The modified starch derivative was prepared by treating starch with an exo-enzyme, such as β-amylase, which requires a non-reducing

end. It was shown in US 4,977,252 that such modified starch derivatives exhibited a reduced tendency of retrogradation upon storage.

However, there is still a need in this technical field for new enzymes, which can be used in the preparation of modified starch derivatives.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that enzymatically modified starch derivatives having, for example, a reduced tendency towards retrogradation and thus being suitable for, e.g., emulsions where a long shelf stability is desirable, may conveniently be produced by means of maltogenic amylases, i.e. enzymes which do not necessarily need a non-reducing end in order to degrade the starch. Furthermore, it has surprisingly been found that some maltogenic amylases have an increased thermostability which opens up the possibility of processing the starch at higher temperatures than usually done by the conventional prior art methods. This in turn means that the gelatinization step and the enzyme-treatment step may be carried out simultaneously, e.g. at a temperature about 85°C-90°C, i.e. two process steps are reduced to one thereby leading to improved process economy as compared to prior art methods.

Thus, in a first aspect the present invention relates to a method for preparing an enzymatically modified starch derivative, the method comprising the following steps:

I) treating a starch derivative with the maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO:1 or a variant thereof, where the variant

a) has maltogenic amylase activity;

b) has at least 70% identity to the amino acids 1-686 shown in SEQ ID NO:1, and

c) has optimum maltogenic amylase activity in the pH range 3.5-7.0; and

II) optionally recovering the enzymatically modified starch derivative.

In a second aspect the present invention relates to a method for preparing an enzymatically modified starch derivative, the method comprising the following steps:

I) treating starch with the maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO:1 or a variant thereof, where the variant

a) has maltogenic amylase activity,

b) has at least 70% identity to the amino acids 1-686 shown in SEQ ID NO: 1, and

c) has optimum maltogenic amylase activity in the pH range 3.5-7.0;

Ia) chemically deriving the enzymatically modified starch; and

II) optionally recovering the enzymatically modified starch derivative.

In a third aspect the present invention relates to an enzymatically modified starch derivative obtainable by the methods of the invention.

In a fourth aspect the present invention relates to an emulsion comprising the enzymatically modified starch derivative of the invention.

Further aspects of the present invention relate to food products, beverage flavour concentrates and flavouring agent comprising an emulsion of the invention.

Still further aspects of the present invention relates to the use of maltogenic amylases, as well as the use of the enzymatically modified starch derivative of the invention.

Other aspects and particulars of the present invention will be apparent from the below description and from the appended claims.

Definitions

When used herein the term "derivative", when used in connection with "starch", is intended to mean that the starch has been chemically derivatized. The starch may be the base starch or it may have been subjected to some degradation either enzymatically or by some chemical treatment, such as acid treatment.

When used herein the term "modified", when used in connection with "starch" or "starch derivative", is intended to mean that that the starch molecule (whether derivatized or not) has been subjected to an enzymatic degradation treatment.

The term "maltogenic amylase activity" is defined herein as the capability of an enzyme to hydrolyse amylose and amylopectin to maltose in the alpha configuration. One Maltogenic Amylase Novo Unit (MANU) is the amount of enzyme which under standard conditions will cleave 1 μmol maltotriose per minute. The standard conditions are 10 mg/ml maltotriose, 37°C, pH 5.0 and 30 min reaction time.

For purposes of the present invention, the degree of identity between two amino acid sequences may be suitably determined according to the method described in Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45, with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The determination may be done by means of a computer program known such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

When used herein the term "mean molecular weight" is intended to mean the mass average molecular weight of the modified starch derivative. Thus, the term "mean molecular weight" means the mass average molecular weight for the "purified" modified starch derivative fraction from which the maltose fraction has been separated.

In the present context the term "beverage flavour concentrate" covers a flavoured syrup base used for soft drinks, i.e. a liquid system containing flavouring oils.

DETAILED DISCLOSURE OF THE INVENTION

The preparation of enzymatically modified starch derivatives according to the invention is described in the following section. A detailed description of suitable enzymes for the purpose is given in subsequent sections.

Methods for preparing enzymatically modified starch derivatives according to the invention

In one embodiment of the present invention, the enzyme treatment is carried out on starches after they have been derivatized. In a preferred embodiment of the invention, the starch is chemically derivatized in such a way that the resulting enzymatically modified starch derivative contains either hydrophobic groups, or groups comprising both hydrophilic and hydrophobic moieties, so as to have emulsifying properties.

Alternatively, the enzyme treatment is carried out before the starch derivative has been prepared.

Methods for preparing such derivatives are disclosed in US 2,661,349 mentioned earlier and incorporated herein by reference. The preferred starch base is a starch alkenyl succinate half ester, wherein the carboxyl group may be present as an acid or as a carboxylate salt. It is noted, however, that any method which yields the desired hydrophobic function or a blend of hydrophobic and hydrophilic functions on the starch molecule, and thereby provides it with emulsifying properties, can be used to prepare the modified starch derivatives herein. Suitable derivatives and methods for producing them are disclosed in US 4,626,288.

The treatment with the maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO: 1 or variants thereof, which do not require a non-reducing end is carried out until at the most 75% by weight of the starch or starch derivative has been degraded to maltose. Dependent on the actual use of the modified starch derivative the degradation is typically carried out until 25-75% by weight, preferably 30-70% by weight, such as 40-70% by weight, in particular 50-70% by weight of the starch or starch derivative has been degraded to maltose.

In preparing the modified starch derivative of the invention, the desired starch base is slurried in water in any proportion needed to achieve the desired enzyme-substrate concentration or calculated to suit the end use. Typically, the mixture is then cooked to gelatinize the starch.

After gelatinization of the starch the temperature and pH of the mixture are then adjusted to the optimums recommended by the manufacturer or supplier for the particular enzyme to be used in preparing the starch product.

Contrary to the disclosure of US 4,977,252, wherein an enzyme having a strict exo-activity is required it has surprisingly been found by the present inventors that the maltogenic amyalses described herein (*vide infra*) are suitable for preparing the modified starch derivatives according to the invention. It has to our best knowledge not been described in the prior art that enzymes having a considerable degree of endo-activity may also be employed for the preparation of modified starches, which in turn have useful emulsifying properties. A detailed description of suitable maltogenic amylases is given in the section entitled "Maltogenic amylases".

The enzyme is permitted to digest the starch base until up to 75% by weight, typically 25-75% by weight, preferably 30-70% by weight, such as 40-70% by weight, in

particular 50-70% by weight of the starch or starch derivative has been degraded to maltose and other smaller oligosachharides, or until the desired end point (i.e., sufficient degradation to provide improved shelf stability of a particular emulsion prepared with the starch) has been reached. The end point may be determined by change in viscosity, by reducing sugar content, by determination of molecular weight, or by any other method known in the art for measuring the level of enzyme degradation of the starch molecule.

The enzyme degradation reaction may be terminated by means of heat, chemical additions, or other methods known in the art for deactivating an enzyme or separating an enzyme from its substrate.

The resulting degraded starch composition may be spray-dried, drum-dried or otherwise recovered in a form suitable for the intended application.

It is to be understood that the invention herein includes any emulsified composition wherein the emulsifying agent is starch which has been enzymatically modified according to the invention. Thus, it is meant to include emulsions comprising a blend of the modified starch and gums or other emulsifying agents.

The modified starch derivatives of the invention may be advantageously employed in any product wherein gum arabic has been used as an emulsifier, stabilizer, or the like, and in any product where high molecular weight, water soluble emulsifiers have been used to form or stabilize emulsions. Thus, it may be used in beverages that are flavoured with oils such as orange or lemon oils, confectionery items, ice cream, other beverages and other food products which require a shelf stable emulsifier.

It may be used in water-and-alcohol based beverages. The modified starch derivatives of the invention may also be used in preparing spray-dried flavour oilswhich are reconstitutable with water to provide flavour emulsions, and in inks, for paper coatings or paper sizing, textiles and other non-food end uses.

In connection with paper sizing or paper coating, it is particularly preferred that the enzymatically modified starch derivative contains only a hydrophilic group (see above).

The applicable starch bases, which may be used in preparing the enzymatically modified starch derivative herein may be derived from any plant source including corn, potato, sweet potato, wheat, rice, sago, tapioca, waxy maize, sorghum, and the like. Also included are the conversion products derived from any of the above bases

including, for example, fluidity or thin-boiling starches prepared by oxidation, mild acid hydrolysis or heat dextrinization, and derivatized starches such as ethers and esters.

As indicated above, the starch base will in many cases be a gelatinized starch (a precooked, non-granular starch). It may also be a fluidity starch converted by mild acid degradation, or heat dextrinization methods that are well known in the art. (See e.g., M. W. Rutenberg, "Starch and Its Modifications" in Handbook of Water-Soluble Gums and Resins, R. L. Davidson, editor, McGraw Hill, Inc., New York, N.Y., 1980, pp. 22-36.) A combination of one or more of these conversion techniques may be used. The conversion is typically carried out before treatment with the hydrophobic/hydrophilic (or merely hydrophobic) reagent and before the maltogenic amylase treatment.

The starch may be derivatized by treatment with any reagent, which confers emulsification properties to the starch. The derivatization may be carried out before or after the enzyme treatment. The reagent should contain a hydrophobic moiety and may contain a hydrophilic moiety. The hydrophobic moiety preferably contains at least 5 carbon atoms, in particular 5 to 24 carbon atoms. Preferably the hydrophobic moiety is derived from a fatty acid, alkyl, alkenyl, aralkyl or aralkenyl group.

As in the preferred embodiment set forth below, the hydrophilic moiety may be contributed by the reagent, or, as in other embodiments, the starch's own hydroxyl groups serve as the hydrophilic moiety and the reagent only contributes a hydrophobic moiety. Other examples of suitable hydrophilic groups include hydroxy ethyl, hydroxy propyl as well as cationic groups.

In a preferred embodiment, the starch is derivatized by reaction with an alkenyl cyclic dicarboxylic acid anhydride by the method taught in US 2,661,349. However, any process for derivatizing starch which yields the desired blend of hydrophobic and hydrophilic functions on the starch molecule, and thereby yields stable emulsification properties, may be used to prepare the enzymatically modified starch derivatives claimed herein.

In general, if a low viscosity emulsifier is desirable, the preferred embodiment is an octenylsuccinate half ester derivative of an amylopectin-containing starch, such as waxy maize.

For other products, any degree of substitution or level of conversion that results in the desired viscosity and emulsification characteristics may be employed. For example, US 4,035,235 discloses a suitable embodiment, comprising a method for producing a lipophilic derivative of starch to be used as an alternative to gum arabic in

encapsulating water insoluble substances, such as volatile flavouring oils and perfumes.

In a preferred embodiment, the next step after preparing the starch derivative is to gelatinize the derivatized starch. The gelatinization process unfolds the starch molecules from the granular structure, thereby permitting the enzyme to more easily and uniformly degrade the outer branches of the starch molecules. After a slurry of the starch base has been gelatinized, the solids, temperature and pH of the slurry are adjusted to provide optimum enzyme activity.

The optimum parameters for enzyme activity will vary depending upon the enzyme used. Thus, the rate of enzyme degradation depends on factors including the type of enzyme used, enzyme concentration, substrate concentration, pH, temperature, the presence or absence of inhibitors and other factors. Depending on the type of enzyme, or its source, various parameters may require adjustment to achieve optimum digestion rate. In general, the preferred enzyme digestion reaction is carried out at the highest solids content that is feasible to facilitate subsequent drying of the starch composition while maintaining optimum reaction rates.

Optimum concentrations of enzyme and substrate are governed by the level of enzyme activity under the applied conditions, e.g. the desired reaction time, and it will be a matter of routine for the skilled person to determine such optimum concentrations. As will be understood by the skilled person the enzyme of choice may be utilized in solution or it may be immobilized on a solid support.

If desired, the reaction may proceed in the presence of buffers to ensure that the pH will be at the optimum level throughout the degradation. Buffers such as acetates, citrates, or the salts of other weak acids are acceptable. Other agents may be used to optimize enzyme activity. The reaction may be carried out in a pH range from about 3-10, such as from about 5-7, depending on the specific enzyme employed. As will be apparent from the below section relating to suitable enzymes for carrying out the reaction, the preferred pH range will be dependent on the specific enzyme chosen for the purpose.

In general, the aqueous starch dispersion should be held, during the enzymatic digestion, at a temperature of about 20°C-100°C. As with other parameters of the enzyme reaction, the preferred and optimum temperature ranges will vary with changes in other parameters such as substrate concentration, pH and other factors affecting enzyme activity, and can be determined by the practitioner.

However, in a particular interesting embodiment of the invention, wherein a thermostable variant of the maltogenic amylase having the amino acid sequence shown in SEQ ID NO: 1 is employed, the enzymatic treatment may be carried out at a temperature above 65°C, in particular at a temperature about or above 70°C, such as about or above 75°C, e.g. about or above 80°C, preferably about or above 85°C, e.g. about or above 90°C, such as about or above 95°C.

As will be apparent from the below sections discussing various enzymes suitable for the purpose of preparing the modified starch derivative according to the invention, it has been found that some variants of the maltogenic variant having the amino acids 1-686 shown in SEQ ID NO: 1 possess altered properties compared to the parent enzyme. Thus, as will be acknowledged by the skilled person, this opens up the possibility of carrying out the starch degradation reactions under "unusual" conditions, i.e. the starch degradation process may be carried out under, for example elevated temperatures, at different pH-values, etc. as compared to the prior art methods.

In a particular interesting embodiment of the invention the enzymatic modification is carried out with a thermostable maltogenic variant at a temperature in the range of 80-100°C, such as in the range of 85-95°C, e.g. about 90°C.

Operating at elevated temperatures such as those described above means that a higher content of dry matter (base starch) can be used in the reaction mixture. Furthermore, microbial contamination of the reaction mixture is likely to be reduced when operating at higher temperatures.

Moreover, the possibility of carrying out the enzyme-treatment step at temperatures in the range of 80-100°C, such as in the range of 85-95°C, e.g. about 90°C means that the gelatinization step and the enzyme-treatment step may be carried out simultaneously thereby avoiding an additional step in the process. Therefore, in a particular interesting embodiment of the invention, the enzymatic treatment is carried out simultaneously with the gelatinization of the starch or starch derivative.

The below sections relating to suitable enzymes for the purposes described herein contains a number of examples of such thermostable enzymes. As not all modifications in a given enzyme lead to an increased thermostability, the present inventors have developed a suitable test, which enables the skilled person to assess the suitability of a given enzyme. Thus, an enzyme which is considered particular useful for preparing the modified starch derivatives according to the invention is a maltogenic amylase variant, which

a) has maltogenic amylase activity;

b) has at least 70% identity to the amino acids 1-686 SEQ ID NO:1;

c) has optimum maltogenic amylase activity in the range pH 3.5-7.0; and

d) shows a residual maltogenic amylase activity of at least 25% after incubation with 1 mM Ca^{++} at pH 4.3, at 80°C for 15 min.

In a highly preferred embodiment of the invention the maltogenic amylase variant shows a residual maltogenic amylase activity of at least 30%, e.g. at least 40%, preferably at least 50%, e.g. at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90% after incubation with 1 mM Ca^{++} at pH 4.3, at 80°C for 15 minutes.

In a particular preferred embodiment of the invention the maltogenic amylase variant shows a residual maltogenic amylase activity of at least 30%, e.g. at least 40%, preferably at least 50%, e.g. at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90% after incubation with 1 mM Ca^{++} at pH 4.3 for 15 minutes at a temperature at 85°C or even at a temperature of 90°C.

Obviously, it is desirable that such variants fulfil the requirements set forth above on at least the stated minimum level, preferably at the stated intermediary level, and most preferred such variants should fulfil the requirements on the stated highest level.

The enzyme reaction is permitted to continue until the desired level of degradation is reached. The progress of enzyme reaction may be measured by various methods. If all critical parameters have been established for achieving a particular starch composition, then the reaction may be allowed to proceed to a predetermined relative end point in time. The end point also may be monitored and defined by measuring the concentration of reducing sugars. The maltose, which is produced by the enzyme, is a reducing sugar, which is easily measured by methods well known in the art. Other techniques such as monitoring the change in viscosity or the change in molecular weight may be used to define the reaction end point.

The degree of starch degradation that is required to substantially improve the low temperature stability of the starch composition is subject to variation. It depends, *i.a.* on the type of starch utilized, the presence and nature of any substituent groups. Obviously, the desired degree of starch degradation is also dependent on the end-use purpose of the modified starch derivative.

Dependent on the desired end use of the product the starch degradation may be continued until a certain mean molecular weight of the resulting modified starch derivative is obtained.

The mean molecular weight may be determined in a number of ways known to the skilled person. Moreover, the mean molecular weight may be determined on the "total" reaction mixture, i.e. the mixture containing the modified starch derivative, maltose as well as small amounts of by-products such as glucose and maltotriose.

Clearly, the mean molecular weight based on the "total" reaction mixture will, due to the rather high content of maltose, be somewhat lower than the mean molecular weight determined on the modified starch derivative as such. One suitable method for determining the mean molecular weight of the modified starch derivative-components in the reaction mixture is Gel Permeation Chromatography (GPC). Other methods which will be known to the person skilled in the art may, however, also be employed. A description of one suitable GPC method is given in the Experimental section herein.

As described previously, when used herein the term "mean molecular weight" is intended to mean the mass average molecular weight of the modified starch derivative. Thus, the term "mean molecular weight" means the mass average molecular weight for the "purified" modified starch derivative fraction from which the maltose fraction has been separated.

In general, the modified starch derivative of the invention should have a mean molecular weight in the range of from 75,000 to 750,000 when determined by the GPC-method described herein. For preparation of emulsions, in particular for preparation of emulsions to be used in food product, such as beverages, the mean molecular weight is preferably in the range of from 100,000 to 600,000, such as in the range of from 100,000 to 500,000 when determined by the GPC-method described herein.

Although emphasis has been put on the application of the modified starch derivative in emulsions, the skilled person will be able to find other areas of application for the modified starch derivatives disclosed herein. For example, the modified starch derivative may also be useful as encapsulating agents such as described in EP 0 913 406, which is hereby incorporated by reference. In such cases, i.e. when the intended use of the modified starch derivative of the invention is as an encapsulating agent, the mean molecular weight should preferably be somewhat lower, such as in the range of from 10,000 to 100,000, preferably in the range of from 20,000 to 50,000.

One further application of the modified starch derivatives of the invention is as a so-called fat-replacer. In such cases the mean molecular weight should preferably be in the range of from 3,000 to 10,000.

After the desired degree of starch degradation has been reached the enzyme may be deactivated. A number of methods for deactivating maltogenic amylases are available and will be known to the person skilled in the art.

The practitioner will recognize that the sequence of steps in the process of this invention may be carried out in any order, and are not limited to the preferred embodiment set forth above. Thus, in another interesting embodiment, the sequence is reversed so that the enzymatic degradation step is completed prior to the derivatization step.

If the end-use application requires purification (recovering) of the starch composition, the maltose and other reaction impurities and by-products may be removed by dialysis, filtration, centrifugation or any other method known in the art for recovering, isolating and concentrating starch compositions.

If a dried starch composition is desired for end use applications, the starch composition may be dehydrated by any method known in the art.

For low viscosity emulsifiers, the reduction in viscosity during enzyme reaction may be used for determining when the desired level of degradation has been reached. Any of the many methods known in the art for measuring viscosity may be used.

Maltogenic amylases

As indicated above, a maltogenic amylase (glucan 1,4-α-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyse amylose and amylopectin to maltose in the alpha-configuration. Furthermore, a maltogenic amylase is able to hydrolyse maltotriose as well as cyclodextrin.

A maltogenic amylase from *Bacillus* (EP 120 693) is commercially available from Novo Nordisk A/S, Denmark and is widely used in the starch industry. It is most active at 60-70°C (Christophersen, C., et al., 1997, Starch, vol. 50, No. 1, 39-45). This particular maltogenic amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1.

Also, this particular maltogenic amylase shares several characteristics with cyclodextrin glucanotransferases (CGTases), including sequence homology (Henrissat B., Bairoch A. 1996) and formation of transglycosylation products (Christophersen, C., et al., 1997, Starch, vol. 50, No. 1, 39-45). Cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19), also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, abbreviated herein as CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins (or CD) of various sizes.

As mentioned above, maltogenic amylase is an enzyme classified in EC 3.2.1.133. The enzymatic activity does not require a non-reducing end on the substrate and the primary enzymatic activity results in the degradation of amylopectin and amylose to maltose and longer maltodextrins. It is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration, and is also able to hydrolyze maltotriose as well as cyclodextrin.

One interesting maltogenic amylase is the amylase cloned from *Bacillus* as described in EP 120 693, which has the amino acid sequence set forth in amino acids 1-686 of SEQ ID NO:1. This maltogenic amylase is encoded in the gene harbored in the *Bacillus* strain NCIB 11837 which has the nucleic acid sequence set forth in SEQ ID NO:1. The three-dimensional structure of the above-mentioned maltogenic amylase is described below.

In general, a preferred maltogenic amylase should have one or more of the following properties:

i) a three dimensional structural homology to the maltogenic amylase shown as amino acids 1-686 of SEQ ID NO:1,
ii) maltogenic amylase activity,
iii) an amino acid sequence having at least 70% identity to the amino acids 1-686 shown in SEQ ID NO: 1, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98 %, or at least 99%,
iv) optimum maltogenic activity in the range pH 3.5-7.0, preferably in the range pH 4-5.5.

Three-dimensional structure of maltogenic amylase

The structure of the maltogenic amylase shown as amino acidas 1-686 of SEQ ID NO:1 is made up of five globular domains, ordered A, B, C, D and E. The domains can be defined as being residues 1-132 and 204-403 for Domain A, residues 133-203 for

Domain B, residues 404-496 for Domain C, residues 497-579 for Domain D, and residues 580-686 for Domain E, wherein the numbering refers to the amino acid sequence in SEQ ID NO: 1. Features of Domains A, B, and C of particular interest are described below.

Domain A

Domain A is the largest domain and contains the active site which comprises a cluster of three amino acid residues, D329, D228 and E256, spatially arranged at the bottom of a cleft in the surface of the enzyme. The structure of Domain A shows an overall fold in common with the α-amylases for which the structure is known, viz. the (beta/alpha) 8 barrel with eight central beta strands (numbered 1-8) and eight flanking a-helices. The β-barrel is defined by McGregor *op. cit.* The C-terminal end of the beta strand 1 is connected to helix 1 by a loop denoted loop 1 and an identical pattern is found for the other loops, although the loops show some variation in size and some can be quite extensive.

The eight central beta-strands in the (beta/alpha) 8 barrel superimpose reasonably well with the known structures of CGTases. This part of the structure, including the close surroundings of the active site located at the C-terminal end of the beta-strands, shows a high degree of identity with CGTases.

In contrast, the loops connecting the beta-strands and alpha helices display a high degree of variation from the known structures of CGTases. These loops constitute the structural context of the active site, and the majority of the contacts to the substrate is found among residues located in these loops. Distinguishing characteristics such as substrate specificity, substrate binding, pH activity profile, substrate cleavage pattern, and the like, are determined by specific amino acids and the positions they occupy in these loops. Domain A contains two calcium binding sites, one of which is homologous to the calcium binding site in CGTases; the other is unique to the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1. The structure of the calcium binding site is discussed further below in the section "Calcium binding sites."

Domain B

Domain B, also referred to as loop 3 of the (beta/alpha) 8 barrel, in comprises amino acid residues 133-203 of the amino acid sequence shown in SEQ ID NO: 1. The structure is partially homologous to the structure of Domain B in CGTases, the most striking difference being the presence of a five amino acid insert corresponding to

positions 191-195 in the amino acid sequence shown in SEQ ID NO: 1 which is not found in the CGTases. This insert is spatially positioned close to the active site residues and in close contact to the substrate.

Domain C

Domain C in the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 comprises amino acid residues 404-496 of the amino acid sequence shown in SEQ ID NO: 1. Domain C is composed entirely of β-strands which form a single 8-stranded sheet structure that folds back on itself, and thus may be described as a β-sandwich structure. One part of the β-sheet forms the interface to Domain A.

Calcium binding sites

The structure of the maltogenic amylase exhibits three calcium-binding sites; that is, three calcium ions are found to be present in the structure. In common with most of the known family 13 structures, one calcium ion is located between the A and B domains. This calcium ion is coordinated by a backbone carbonyl atom from Gln184 and His232, sidechain atoms from Asp198, a sidechain atom from Asn131, and three water molecules V8.

A second calcium ion is located in the A domain and is common to CGTases, but not found in α-amylases. The calcium ion is coordinated by a backbone carbonyl atom from Gly48 and Asp23, a sidechain atom from Asp50, a sidechain atom from Asp21, a sidechain atom from Asn26, and a sidechain atom from Asn27, and one water molecule.

The third calcium ion is located in the A Domain and is unique to the maltogenic amylase shown as amino acids 1-686 of SEQ ID NO:1. The coordination comprises a backbone carbonyl atom from Asn77, sidechain atoms from Glu102, a sidechain atom from Asp79, a sidechain atom from Asp76, a sidechain atom from Glu101, and one water molecule.

Substrate Binding Site

Parts of the loop discussed above in the context of domains A and B are of particular interest for substrate interaction and active site reactivity. In particular, in domain A, residues 37-45 in loop 1, residues 261-266 in loop 5, residues 327-330 in loop 7 and residues 370-376 in loop 8; in domain B, residues 135-145 in loop 3, residues 173-180 and 188-196 in loop 3, wherein residue positions correspond to the amino acids in the amino acid sequence in SEQ ID NO: 1.

Without being limited to any theory, it is presently believed that binding between a substrate and an enzyme is supported by favorable interactions found within a sphere of 4 to 6 Å between the substrate molecule and the enzyme, such as hydrogen bonds and/or strong electrostatic interaction. The following residues of SEQ ID NO: 1 are within a distance of 6 Å of the substrate HEX and thus believed to be involved in interactions with said substrate: 44, 89, 90, 92, 93, 127, 129, 132, 135, 177, 178, 188, 191, 194, 196, 226, 228, 229, 230, 231, 232, 256, 258-261, 288, 328, 329, 371, 372, 373, 376, and 690.

The following residues of SEQ ID NO:1 are within a distance of 4 Å of the substrate HEX and thus believed to be involved in interactions with said substrate: 90, 92, 93, 129, 132, 177, 188, 189, 190, 191, 196, 226, 228, 229, 231, 232, 256, 258, 259, 260, 261, 328, 329, 372, 376, and 690.

Homology building of maltogenic amylases

A model structure of a maltogenic amylase can be built using the Homology program or a comparable program, e.g., Modeller (both from Molecular Simulations, Inc., San Diego, CA). The principle is to align the sequence of the maltogenic amylase with the known structure with that of the maltogenic amylase for which a model structure is to be constructed. The structurally conserved regions can then be built on the basis of consensus sequences. In areas lacking homology, loop structures can be inserted, or sequences can be deleted with subsequent bonding of the necessary residues using, e.g., the program Homology. Subsequent relaxing and optimization of the structure should be done using either Homology or another molecular simulation program, e.g., CHARMm from Molecular Simulations.

Maltogenic amylase variants with altered thermostability and/or altered temperature dependent activity profile

As explained above, one of the advantages of using either the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 or variants thereof in the starch processing procedure is the possibility of carrying out the reaction at elevated temperatures. Accordingly, variants which are particularly interesting for the purposes described herein are such variants which

a) have maltogenic amylase activity,

b) have at least 70% identity to the amino acids 1-686 shown in SEQ ID NO: 1,

c) have optimum maltogenic amylase activity in the range pH 3.5-7.0, and

d) show a residual maltogenic amylase activity of at least 25% after incubation with 1 mM Ca^{++} at pH 4.3, 80°C for 15 minutes.

Preferably, the variant should possess as high a thermostability as possible and, accordingly, variants which are particular preferred are variants which fulfill the criteria set forth under d) above at an even higher level, e.g. variants which show a residual maltogenic amylase activity of at least 30%, e.g. at least 40%, preferably at least 50%, e.g. at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90% after incubation with 1 mM Ca^{++} at pH 4.3, 80°C for 15 minutes.

The structure of the maltogenic amylase contains a number of unique internal cavities which may contain water and a number of crevices. In order to increase the thermostability of the variant it may be desirable to reduce the number or size of cavities and crevices, e.g., by introducing one or more hydrophobic contacts, preferably achieved by introducing amino acids with bulkier side groups in the vicinity or surroundings of the cavity. For instance, the amino acid residues to be modified are those which are involved in the formation of the cavity.

It will be understood that the cavity or crevice is identified by the amino acid residues surrounding said cavity or crevice, and that modification of said amino acid residues are of importance for filling or reducing the size of said cavity or crevice. Preferably, the modification is a substitution with a bulkier amino acid residue, i.e. one with a greater side chain volume. For example, all the amino acids are bulkier than Gly, whereas Tyr and Trp are bulkier than Phe. The particular amino acid residues referred to below are those which in a crystal structure have been found to flank the cavity or crevice in question.

Thus, in a preferred embodiment of the invention the maltogenic amylase to be used for producing the enzymatically modified starch derivative according to the invention, comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

L51, L71, S72, V74, L75, L78, T80, L81, G83, T84, D85, N86, T87, G88, Y89, H90, G91, T94, R95, D96, F97, V114, I125, V126, T134, G157, Y167, F168, H169, H170, N171, G172, D173, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, T189, D190, A192, G193, F194, S195, L196, L217, S235, G236,

V254, V279, V281, L286, V289, I290, V308, L321, I325, D326, L343, F349, S353, I359, I405, L448, Q449, L452, I470, G509, V515, S583, G625, L627, L628 or A670.

In a more preferred embodiment, the variant of a maltogenic amylase comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

L217 in combination with L75 (e.g. L217F/Y in combination with L75F/Y), L51W, L75F/Y, L78I, G88A/V/T, G91T/S/V/N, T94V/I/L, V114V/I/L, I125L/M/F/Y/W, V126I/L, T134V/I/L/M/F/Y/W, G157A/V/I/L, L217V/I/M/F/Y/W, S235I/L/M/F/Y/W, G236A/V/I/L/M/F/Y/W, V254I/L/M/F/Y/W, V279M/I/L/F, V281I/L/M/F/Y/W, L286F, V289I/L/R, I290M/L/F, V308I/L/M/F/Y/W, L321I/M/F/Y/W, I325L/M/F/Y/W, D326E/Q, L343M/F/Y/W, F349W/Y, S353V/I/L, I359L/M/F/Y/W, I405M/L/Y/F/W, L448Y, Q449Y, L452M/Y/F/W, I470M/L/F, G509A/V/I/L/M/S/T/D/N, V515I/L, S583V/I/L/V, G625A/V/I/L/M/F/Y/W, L627M/F/Y, L628M/I/F/Y/W, A670V/I/L/M/F/Y/W, L71I, S72C, V74I, L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, G83A/S/T/N/Q/E/D/R/H/L, T84S/A/N/D/G, D85A/T/S/N/G, N86Q/E/D/Y/H/K, T87S/I, G88A/S/T, Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q, D96N/V/Q/I, F97Y, Y167F/R/C, F168Y, H169N/Q/K, H170N/Q/K, N171D/E/Q/H/R/K/G, G172A/T/S, D173N/S/T/Y/R/G, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W, Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L, F188Y/L/I/H/N, T189N/D/A/S/H/Y/G, D190E/Q/H/N/K, A192T/D/E/N/K, G193A/S/T, F194Y, S195N/D/E/R/K/G, and L196I.

Similar substitutions may be introduced in equivalent positions of other maltogenic amylases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

Maltogenic amylase variants with altered stability

In general, variants having improved (increased) stability may be obtained by stabilization of calcium binding, substitution with proline, substitution of histidine with another amino acid, introduction of an interdomain disulfide bond, removal of a deamidation site, altering a hydrogen bond contact, filling in an internal structural cavity with one or more amino acids with bulkier side groups, introduction of interdomain interactions, altering charge distribution, helix capping, or introduction of a salt bridge.

Calcium binding

Variants which are contemplated to be suitable for the starch processing procedure described herein are variants which have an altered stability due to an altered stabilization of calcium (Ca^{2+}) binding. The enzyme variant may have altered thermostability or pH dependent stability, or it may have maltogenic amylase activity in the presence of a lower concentration of calcium ion. It is presently believed that amino acid residues located within 10 Å from a calcium ion are involved in or are of importance for the Ca^{2+} binding capability of the enzyme.

The amino acid residues found within a distance of 10 Å from the Ca^{2+} binding sites of the maltogenic amylase with the amino acid sequence set forth in SEQ ID NO: 1 are as follows:

16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 29, 30, 31, 32, 33,35, 36, 40, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 73, 74, 75, 76, 77, 78, 79, 80, 81, 87, 88, 89, 91, 93, 94, 95, 96, 99, 100, 101, 102, 103, 104, 105, 109, 129, 130, 131, 132, 133, 134, 145, 150, 167, 168, 169, 170, 171, 172, 174, 177, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 196, 197, 198, 199, 200, 201, 202, 206, 210, 228, 229, 230, 231, 232, 233, 234, 235, 237, 378, and 637.

In order to construct a variant according to this aspect of the invention it is desirable to substitute at least one of the above mentioned amino acid residues, which is determined to be involved in a non-optimal calcium binding, with any other amino acid residue which improves the Ca^{2+} binding affinity of the variant enzyme. Accordingly, one method of constructing a variant of a parent maltogenic amylase wherein said variant has a stabilised Ca^{2+} binding as compared to said parent amylase comprises:

i) identifying an amino acid residue within 10 Å from a Ca^{2+} binding site of a maltogenic amylase in a model of the three-dimensional structure of said amylase which, from structural or functional considerations, is determined to be responsible for a non-optimal calcium ion interaction;

ii) constructing a variant in which said amino acid residue is substituted with another amino acid residue which, from structural or functional considerations, is determined to be important for establishing an altered Ca^{2+} binding affinity; and

iii) testing the Ca^{2+} binding of the resulting maltogenic amylase variant.

Substituting an amino acid residue responsible for non-optimal calcium ion interaction with another residue may alter a calcium ion binding interaction of the enzyme. For instance, the amino acid residue in question may be selected on the basis of one or more of the following objectives:

a) to obtain an improved interaction between a calcium ion and an amino acid residue as identified from the structure of the maltogenic amylase. For instance, if the amino acid residue in question is exposed to a surrounding solvent, it may be advantageous to increase the shielding of said amino acid residue from the solvent so as to stabilize the interaction between said amino acid residue and a calcium ion. This can be achieved by substituting said residue, or an amino acid residue in the vicinity of said residue contributing to the shielding, with an amino acid residue with a bulkier side group or which otherwise results in an improved shielding effect.

b) to stabilize a calcium binding site, for instance by stabilizing the structure of the maltogenic amylase, e.g. by stabilizing the contacts between two or more of the five domains or stabilizing one or more of the individual domains as such. This may, e.g., be achieved by providing for a better coordination to amino acid side chains, which may, e.g., be obtained by substituting an N residue with a D residue and/or a Q residue with an E residue, e.g. within 10 Å, and preferably within 3 or 4 Å, of a calcium binding site.

c) to improve the coordination between the calcium ion and the calcium binding residues, e.g., by improving the interaction between the ion and the coordinating residues or increasing the number of sidechain coordinations by substituting a coordinating water with an amino acid sidechain.

d) replace water by a coordinating calcium amino acid residue.

Preferably, the amino acid residue to be modified is located within 8 Å of a Ca^{2+} ion, in particular within 5 Å of a Ca^{2+} ion.

In a preferred embodiment, the variant of a maltogenic amylase having an altered Ca^{2+} binding as compared to the parent maltogenic amylase comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

D17, A30, S32, R95, H103, N131, Q201, I174, H169, V74, L75, L78, T80, L81, T87, G88, Y89, H90, G91, T94, R95, D96, F97, Y167, F168, H169, H170, N171, G172,

D173, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, and/or T189.

In more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

D17E/Q, A30M/L/A/V/I/E/Q, S32D/E/N/Q, R95M/L/A/V/I/E/Q, H103Y/N/Q/D/E, N131D, Q201E, I174E/Q, H169N/D/E/Q, V74I, L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, T87S/I, G88A/S/T, Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q, D96N/V/Q/I, F97Y, Y167F/R/C, F168Y, H169N/Q/K, H170N/Q/K, N171D/E/Q/H/R/K/G, G172A/T/S, D173N/S/T/Y/R/G, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W, Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L, F188Y/L/I/H/N, and/or T189N/D/A/S/H/Y/G.

In another preferred embodiment of the invention with respect to altering the Ca^{2+} binding of a maltogenic amylase the partial sequence N28-P29-A30-K31-S32-Y33-G34 as set forth in SEQ ID NO: 1 is modified.

Similar substitutions may be introduced in equivalent positions of other maltogenic amylases. Modifications of particular interest are any combination of one or more of the above with any of the other modifications disclosed herein.

Other substitutions

Variants with improved stability of the enzyme can be achieved by improving existing or introducing new interdomain and intradomain contacts. Such improved stability can be achieved by the modifications listed below.

The maltogenic amylase having the amino acid sequence shown in SEQ ID NO: 1 may be stabilized by the introduction of one or more interdomain disulfide bonds. Accordingly, another preferred embodiment of the present invention relates to a variant of a parent maltogenic amylase which has improved stability and at least one more interdomain disulfide bridge as compared to said parent, wherein said variant comprises a modification in a position corresponding to at least one of the following pairs of positions in SEQ ID NO: 1: G236 + S583, G618 + R272, T252 + V433 and/or A348 + V487.

In a more preferred embodiment, the substitution corresponds to at least one of the following pairs: G236C + S583C, G618C + R272C, T252C + V433C and/or A348C + V487C.

In another interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability and an altered interdomain interaction as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

i) F143, F194, L78;
ii) A341, A348, L398, I415, T439, L464, L465;
iii) L557;
iv) S240, L268;
v) Q208, L628;
vi) F427, Q500, N507, M508, S573; and/or
vii) I510, V620.

In a more preferred embodiment, the substitution corresponds to at least one of the following sets:

i) F143Y, F194Y, L78Y/F/W/E/Q;
ii) A341S/D/N, A348V/I/L, L398E/Q/N/D, I415E/Q, T439D/E/Q/N, L464D/E, L465D/E/N/Q/R/K;
iii) L557Q/E/N/D;
iv) S240D/E/N/Q, L268D/E/N/Q/R/K;
v) Q208D/E/Q, L628E/Q/N/D;
vi) F427E/Q/R/K/Y, Q500Y, N507Q/E/D, M508K/R/E/Q, S573D/E/N/Q; and/or
vii) I510D/E/N/Q/S, V620D/E/N/Q.

In a further interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability and one or more salt bridges as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1: N106, N320 and Q624.

In a more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1: N106R, N320E/D and/or Q624E.

In a still further interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

K40, V74, S141, T142, F188, N234, K249, D261, D261, L268, V279, N342, G397, A403, K425, S442, S479, S493, T494, S495, A496, S497, A498, Q500, K520, A555 and N595.

In a more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 1:

V74P, S141P, N234P, K249P, L268P, V279P, N342P, G397P, A403P, S442P, S479P, S493P, T494P, S495P, A496P, S497P, A498P, Q500P, and/or A555P.

Other preferred substitutions are K40R, T142A, F188I/L, D261G, K425E, K520R, and/or N595I.

Analogously, it may be preferred that one or more histidine residues present in the parent maltogenic amylase is or are substituted with a non-histidine residues such as Y, V I, L, F, M, E, Q, N, or D. Accordingly, in another preferred embodiment, the variant of a maltogenic amylase comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1: H103, H220, and H344

In a more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1: H103Y/V/I/L/F/Y, H220Y/L/M, and H344E/Q/N/D/Y.

It may be preferred that one or more asparagine or glutamine residues present in the parent maltogenic amylase is or are substituted with a residue lacking the amide on the side chain. Accordingly, in another preferred embodiment, the variant of the maltogenic

amylase having the amino acid sequence shown as SEQ ID NO:1 comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Q13, N26, N77, N86, N99, Q119, N120, N131, N152, N171, N176, N187, Q201, N203, N234, Q247, N266, N275, N276, N280, N287, Q299, N320, N327, N342, Q365, N371, N375, N401, N436, N454, N468, N474, Q500, N507, N513, Q526, N575, Q581, N621, Q624 and N664.

In a more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

Q13S/T/A/V/L/I/F/M, N26S/T/A/V/L/I, N77S/T/A/V/L/I, N86S/T/A/V/L/I, N99T/S/V/L, Q119T/S, N120S/T/A/V/L/I, N131S/T/A/V/L/I, N152T/S/V/L, N171Y/D/S/T, N176S/T/A/V/L/I, N187S/T/A/V/L/I, Q201S/T/A/V/L/I/F/M, N203D/S/T/A/V/L/I, N234S/T/A/V/L/I, Q247S/T/A/V/L/I/F/M, N266S/T/A/V/L/I, N275S/T/A/V/L/I, N276S/T/A/V/L/I, N280S/T/A/V/L/I, N287S/T/A/V/L/I, Q299L/T/S, N320S/T/A/V/L/I, N327S/T/A/V/L/I, N342S/T/A/V/L/I, Q365S/T/A/V/L/I, N371S/T/A/V/L/I, N375S/T/A/V/L/I, N401S/T/A/V/L/I, N436S/T/A/V/L/I, N454D/S/T/A/V/L/I, N468D/S/T/A/V/L/I, N474D/S/T/A/V/L/I, Q500S/T/A/V/L/I/F/M, N507S/T/A/V/L/I, N513S/T/A/V/L/I, Q526 D/S/T/A/V/L/I, N575S/T/A/V/L/I, Q581S/T/A/V/L/I/F/M, N621S/T/A/V/L/I Q624S/T/A/V/L/I/F/M and N664D/S/T/A/V/L/I.

In a further interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability and improved hydrogen bond contacts as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

I16, L35, M45, P73, D76, D79, A192, I100, A148, A163+G172, L268, V281, D285, L321, F297, N305, K316, S573, A341, M378, A381, F389, A483, A486, I510, A564, F586, K589, F636, K645, A629, and/or T681.

In a preferred embodiment, the modification corresponds to one or more of the following:

I16T/D/N, L35Q, M45K, P73Q, D76E, D79E/Y, A192S/D/N, I100T/S/D/N/E/Q, A148D/N/E/Q/S/T/R/K, A163Y+G172S/D/N, L268R/K, V281/Q, D285R/K, L321Q,

F297N/D/Q/E, N305K/R, K316N/D, S573N/D, A341R/K, M378R/K, A381S/D/N, F389Y, A483S/D/N, A486Q/E, I510R/K, A564S/D/N, F586S/D/N, K589S/D/Q/N, F636Y, K645T, A629N/D/E/Q, and/or T681D/N/E/Q/S.

Similar substitutions may be introduced in equivalent positions of other maltogenic amylases. Substitutions of particular interest are any combination of one or more of the above with any of the other modifications disclosed herein.

Before actually constructing a maltogenic amylase variant to achieve any of the above objectives, it may be convenient to evaluate whether or not the contemplated amino acid modification can be accommodated into the maltogenic amylase structure, e.g. into a model of the three-dimensional structure of the parent maltogenic amylase.

Maltogenic amylase variants with altered pH dependent activity profile

It is contemplated that variants having an altered pH dependent activiyt profile may also be suitable for the purposes described herein. The pH dependent activity profile can be changed by changing the pKa of residues within 10 Å of the active site residues of the maltogenic amylase. Changing the pKa of the active site residues is achieved, e.g., by changing the electrostatic interaction or hydrophobic interaction between functional groups of amino acid side chains of a given amino acid residue and its close surroundings. To obtain a higher activity at a higher pH, negatively charged residues are placed near a hydrogen donor acid, whereas positively charged residues placed near a nucleophilic acid will result in higher activity at low pH. Also, a decrease in the pKa can be obtained by reducing the accessibility of water or increasing hydrophobicity of the environment.

Thus, a variant having an altered pH dependent activity profile as compared to the parent enzyme, may be obtained by the following method:

i) identifying an amino acid residue within 15 Å from an active site residue of a maltogenic amylase in the three-dimensional structure of said parent maltogenic amylase, in particular 10 Å from an active site residue, wherein said amino acid residue is contemplated to be involved in electrostatic or hydrophobic interactions with an active site residue;

ii) substituting, in the structure, said amino acid residue with an amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue, and evaluating the accommodation of the amino acid residue in the structure,

iii) optionally repeating step i) and/or ii) recursively until an amino acid substitution has been identified which is accommodated into the structure,

iv) constructing a maltogenic amylase variant resulting from steps i) and ii), and optionally iii), and testing the pH dependent enzymatic activity of said variant.

In a preferred embodiment, the variant of a maltogenic amylase having an altered pH dependent activity profile as compared to the parent maltogenic amylase comprises a modification of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

D127, V129, F188, A229, Y258, V281, F284, T288, N327, M330, G370, N371, D372, L71, S72, V74, L75, L78, T80, L81, G83, T84, D85, N86, T87, G88, Y89, H90, G91, T94, R95, D96, F97, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, T189, D190, A192, G193, F194, S195, and L196.

In more preferred embodiment, the variant comprises a modification corresponding to one or more of the following modifications in the amino acid sequence set forth in SEQ ID NO: 1: D127N/L, V129S/T/G/V, F188E/K/H, A229S/T/G/V, Y258E/D/K/R/F/N, V281L/T, F284K/H/D/E/Y, T288E/K/R, N327D, M330L/F/I/D/E/K, G370N, N371D/E/G/K, D372N/V, L71I, S72C, V74I, L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, G83A/S/T/N/Q/E/D/R/H/L, T84S/A/N/D/G, D85A/T/S/N/G, N86Q/E/D/Y/H/K, T87S/I, G88A/S/T, Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q, D96N/V/Q/I, F97Y, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W, Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L, F188Y/L/I/H/N, T189N/D/A/S/H/Y/G, D190E/Q/H/N/K, A192T/D/E/N/K, G193A/S/T, F194Y, S195N/D/E/R/K/G, and L196I.

Similar modifications may be introduced in equivalent positions of other maltogenic amylases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

Maltogenic amylase variants with an altered cleavage pattern

For the purposes of preparing the enzymatically modified starch and starch derivatives described herein it may be desirable to change cleavage pattern of the applied enzyme, i.e. it may be desirable to change the cleavage pattern, for example, so as to form higher amounts of higher oligosaccharides.

A variant of a parent maltogenic amylase in which the substrate cleavage pattern is altered as compared to said parent may be constructed by a method which comprises:

i) identifying the substrate binding area of the parent maltogenic amylase in a model of the three-dimensional structure, e.g. within a sphere of 4 Å from the substrate binding site as defined in the section above entitled "Substrate Binding Site";

ii) substituting in the model one or more amino acid residues of the substrate binding area of the cleft identified in i) which is or are believed to be responsible for the cleavage pattern of the parent with another amino acid residue which from structural or functional considerations is believed to result in an altered substrate cleavage pattern, or deleting one or more amino acid residues of the substrate binding area contemplated to introduce favorable interactions to the substrate or adding one or more amino acid residues to the substrate binding area contemplated to introduce favorable interactions to the substrate; and

iii) constructing a maltogenic amylase variant resulting from step ii) and testing the substrate cleavage pattern of the variant.

Thus, examples of variants having an altered cleavage pattern which are considered to be useful for the purposes described herein are variants comprising a modification in a position corresponding to one or both of the following positions in SEQ ID NO: 1: V281 and/or A629.

In a preferred embodiment, the variant comprises a modification corresponding to: V281Q and/or A629N/D/E/Q.

Similar modifications may be introduced in equivalent positions of other maltogenic amylases. Substitutions of particular interest are any combination of one or both of the above with any of the other modifications disclosed herein.

Maltogenic amylase variants with improved ability to reduce retrogradation of starch

Obviously, interesting variants also include variants having improved ability to reduce the retrogradation of starch. Preferred variants comprise a modification at one or more positions corresponding to the following amino acid residues in SEQ ID NO: 1: A30,

K40, N115, T142, F188, T189, P191, A192, G193, F194, S195, D261, N327, K425, K520 and N595.

In a more preferred embodiment, the variant comprises one or more modifications corresponding to the following in SEQ ID NO: 1: A30D, K40R, N115D, T142A, F188L, T189Y, Δ (191-195), D261G, D261G, N327S, K425E, K520R and N595I.

Nomenclature for amino acid modifications

The nomenclature used herein for defining mutations is essentially as described in WO 92/05249. Thus, F188H indicates a substitution of the amino acid F (Phe) in position 188 with the amino acid H (His). V129S/T/G/V indicates a substitution of V129 with S, T, G or V. Δ (191-195) or Δ (191-195) indicates a deletion of amino acids in positions 191-195. 192-A-193 indicates an insertion of A between amino acids 192 and 193.

Hybridization

Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al., 1989) for 10 min, and prehybridization of the filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

Methods of preparing variants of maltogenic amylases

Cloning a DNA sequence encoding a maltogenic amylase variant

The DNA sequence encoding a parent maltogenic amylase may be isolated from any cell or microorganism producing the maltogenic amylase in question, using various methods well known in the art, for example, from the *Bacillus* strain NCIB 11837.

First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the maltogenic amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled oligonucleotide probes may be synthesised and used to identify maltogenic amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify maltogenic amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Another method for identifying maltogenic amylase-encoding clones involves inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for maltogenic amylase, thereby allowing clones expressing maltogenic amylase activity to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin, wherein the fragments correspond to various parts of the entire DNA sequence, in accordance with techniques well known in the art. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed Mutagenesis

Once a maltogenic amylase-encoding DNA sequence has been isolated, and desirable sites for modification identified, modifications may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired modification sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the maltogenic amylase-encoding sequence, is created in a vector carrying the maltogenic amylase gene. Then the synthetic nucleotide, bearing the desired modification, is annealed to a

homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple modifications by performing minor alterations of the cassette. However, an even greater variety of modifications can be introduced at any one time by the Morinaga method because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing modifications into a maltogenic amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves a 3-step generation of a PCR fragment containing the desired modification introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the modification may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent maltogenic amylase may be conveniently performed by use of any method known in the art.

In relation to the above, one method for generating a variant of a parent maltogenic amylase, wherein the variant exhibits increased stability at low pH and at low calcium concentration relative to the parent, comprises:

(a) subjecting a DNA sequence encoding the parent maltogenic amylase to random mutagenesis,
(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
(c) screening for host cells expressing a maltogenic amylase variant which has an altered property relative to the parent maltogenic amylase.

Step (a) of the above method of the invention is preferably performed using doped primers, as described in the working examples herein (*vide infra*).

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting

the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the maltogenic amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and modification in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% modifications in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent maltogenic amylase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the maltogenic amylase by, e.g., transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent maltogenic amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harbored in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent maltogenic amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

For region-specific random mutagenesis with a view to improving the stability of calcium binding of a parent maltogenic amylase, codon positions corresponding to the following amino acid residues from the amino acid sequence set forth in SEQ ID NO: 1 may appropriately be targeted:

Residues:Regions:
16-33, 35-36, 40: 16-40
46-54, 56: 46-56
73-81: 73-81
87-89, 91, 93-96, 99-105, 109: 87-109
129-134, (145, 150): 129-134
167-172, 174, 177, 180-189: 167-189
196-202, 206-210: 196-210
228-235, 237: 228-237
378
637

With a view to achieving improved binding of a substrate, i.e., improved binding of a carbohydrate species, such as amylose or amylopectin, by a maltogenic amylase variant with a modified, e.g. higher, substrate specificity and/or a modified, e.g. higher, specificity with respect to cleavage, i.e. hydrolysis, of the substrate, it appears that the following codon positions in the following regions of the amino acid sequence shown in

SEQ ID NO: 1, may particularly appropriately be targeted for modification by region-specific mutagenesis: 70-97, 127-143, 174-198, 226-233, 255-270, 282-292, 324-331, 370-376.

For region-specific random mutagenesis with a view to altering the substrate specificity and/or the pH dependent activity profile, the following regions of SEQ ID NO: 1 may be targeted: 70-97, 174-198.

For random mutagenesis with a view to improving the thermostability, the residues and regions described above for filling internal holes, improved Ca binding, interdomain and intradomain contacts, helix capping, proline substitution, histidine substitution etc. may be targeted. In addition, the following regions may be targeted with a view to improving the thermostability: 70-109, 167-200.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme

2. Decide on mutation sites and nonmutated sites in the selected region

3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed

4. Select structurally reasonable mutations

5. Adjust the residues selected by step 3 with regard to step 4.

6. Analyse by use of a suitable dope algorithm the nucleotide distribution.

7. If necessary, adjust the wanted residues to genetic code realism, e.g. taking into account constraints resulting from the genetic code, e.g. in order to avoid introduction of stop codons; the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted

8. Make primers

9. Perform random mutagenesis by use of the primers

10. Select resulting amylase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One such algorithm is described by Tomandl, D. et al., 1997, Journal of Computer-Aided Molecular Design 11:29-38. Another algorithm is DOPE (Jensen, LJ, Andersen, KV, Svendsen, A, and Kretzschmar, T (1998) Nucleic Acids Research 26:697-702).

Expression of maltogenic amylase variants

The construction of the variant of interest is accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth. This is described in detail further below.

A DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in the form of a protein or polypeptide, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an maltogenic amylase variant may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a maltogenic amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of

E.coli, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes, etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the maltogenic amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding maltogenic amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a maltogenic amylase variant. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the maltogenic amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The maltogenic amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Testing of maltogenic amylase variants

Maltogenic amylase variants produced by any of the methods described above may be tested, either prior to or after purification, for amylolytic activity in a screening assay which measures the ability of the variant to degrade starch. The screening in step 10 in the above-mentioned random mutagenesis method of the invention may be conveniently performed by use of a filter assay based on the following procedure: A microorganism capable of expressing the mutated maltogenic amylase of interest is incubated on a suitable medium and under suitable conditions for secretion of the enzyme, the medium being covered with two filters comprising a protein-binding filter placed under a second filter exhibiting a low protein binding capability. The microorganism is grown on the second, top filter. Subsequent to the incubation, the bottom protein-binding filter comprising enzymes secreted from the microorganism is separated from the second filter comprising the microorganism. The protein-binding filter is then subjected to screening for the desired enzymatic activity, and the corresponding microbial colonies present on the second filter are identified. The first filter used for binding the enzymatic activity may be any protein-binding filter, e.g., nylon or nitrocellulose. The second filter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins, e.g., cellulose acetate or Durapore™.

Screening consists of treating the first filter to which the secreted protein is bound with a substrate that allows detection of the α-amylase activity. The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity. The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents. For example, α-amylase activity can be detected by Cibacron Red labelled amylopectin, which is immobilized in agarose. α-amylase activity on this substrate produces zones on the plate with reduced red color intensity.

To screen for variants with increased stability, the filter with bound maltogenic amylase variants can be pretreated prior to the detection step described above to inactivate

variants that do not have improved stability relative to the parent maltogenic amylase. This inactivation step may consist of, but is not limited to, incubation at elevated temperatures in the presence of a buffered solution at any pH from pH 2 to 12, and/or in a buffer containing another compound known or thought to contribute to altered stability e.g., surfactants, EDTA, EGTA, wheat flour components, or any other relevant additives. Filters so treated for a specified time are then rinsed briefly in deionized water and placed on plates for activity detection as described above. The conditions are chosen such that stabilized variants show increased enzymatic activity relative to the parent after incubation on the detection media.

To screen for variants with altered thermostability, filters with bound variants are incubated in buffer at a given pH (e.g., in the range from pH 2-12) at an elevated temperature (e.g., in the range from 50°-110°C) for a time period (e.g., from 1-20 minutes) to inactivate nearly all of the parent maltogenic amylase, rinsed in water, then placed directly on a detection plate containing immobilized Cibacron Red labelled amylopectin and incubated until activity is detectable. Similarly, pH dependent stability can be screened for by adjusting the pH of the buffer in the above inactivation step such that the parent maltogenic amylase is inactivated, thereby allowing detection of only those variants with increased stability at the pH in question. To screen for variants with increased calcium-dependent stability calcium chelators, such as ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), is added to the inactivation buffer at a concentration such that the parent maltogenic amylase is inactivated under conditions further defined, such as buffer pH, temperature or a specified length of incubation.

The variants may be suitably tested by assaying the starch-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying starch-degrading host cells as described above. Further testing in regard to altered properties, including specific activity, substrate specificity, cleavage pattern, thermoactivation, thermostability, pH dependent activity or optimum, pH dependent stability, temperature dependent activity or optimum, transglycosylation activity, stability, and any other parameter of interest, may be performed on purified variants in accordance with methods known in the art as described below.

Degradation of β-limit dextrin by maltogenic amylase:

Another important parameter in the evaluation of the substrate specificity of maltogenic amylase variants may be the degree to which such enzymes are capable of degrading starch that has been exhaustively treated with the exoglycosylase β-amylase. To screen

for variants which show patterns of degradation on such a substrate differing from the patterns produced by the parent maltogenic amylase the following assay is performed: β-limit dextrin is prepared by incubating 25 ml 1% amylopectin in McIlvane buffer (48.5 mM citrate and 193 mM sodium phosphate pH 5.0) with 24 µg/ml β-amylase overnight at 30°C. Unhydrolysed amylopectin (i.e., β-limit dextrin) is precipitated with 1 volume 98% ethanol, washed and redissolved in water. 1 ml β-limit dextrin is incubated with 18 µl enzymes (at 2.2 mg/ml) and 100 µl 0.2 M citrate-phosphate pH 5.0 for 2 hrs at 30°C and analysed by HPLC as described above. Total hydrolysis of β-limit dextrin is carried out in 2M HCl at 95°C. The concentration of reducing ends is measured by methods known in the art.

Calcium binding affinity

Unfolding of maltogenic amylases by exposure to heat or to denaturants such as guanidine hydrochloride is accompanied by a decrease in fluorescence, and oss of calcium ions leads to unfolding. Thus, the affinity of a maltogenic amylase variant for calcium can be measured by fluorescence measurements before and after incubation of the variant (e.g., at a concentration of 10 mg/ml) in a buffer (e.g., 50 mM HEPES, pH 7) with different concentrations of calcium (e.g., in the range from 1 mM-100 mM) or of EGTA (e.g., in the range from 1-1000 mM) for a sufficiently long period of time (such as 22 hours at 55°C).

The measured fluorescence, *F*, is composed of contributions form the folded and unfolded forms of the enzyme. The following equation can be derived to describe the dependence of *F* on calcium concentration ([Ca]):

$$F = [Ca]/(K_{diss} + [Ca])(a_N - b_N\log([Ca])) + K_{diss}/(K_{diss} + [Ca])(a_U - b_U\log([Ca]))$$

where a_N is the fluorescence of the native (folded) form of the enzyme, b_N is the linear dependence of a_N on the logarithm of the calcium concentration (as observed experimentally), a_U is the fluorescence of the unfolded form and b_U is the linear dependence of a_U on the logarithm of the calcium concentration. K_{diss} is the apparent calcium binding constant for an equilibrium process as follows:

$$N\text{-}Ca \leftrightarrow U + Ca \text{ (N = native enzyme; U = unfolded enzyme)}$$

In fact, unfolding proceeds extremely slowly and is irreversible. The rate of unfolding is dependent on calcium concentration, and such dependency for a given enzyme

provides a measure of the calcium binding affinity of the enzyme. By defining a standard set of reaction conditions (e.g., 22 hours at 55°C), a meaningful comparison of K_{diss} for different maltogenic amylase variants can be made.

Determination of mean molecular weight of enzymatically modified starch derivatives

The mean molecular weight of the fraction containing the enzymatically modified starch derivatives is estimated by Gel Permeation Chromatography (GPC) of the sample obtained after enzymatic treatment. Typically, this result in an elution profile containing (at least) two peaks; one peak containing the modified starch derivative fraction, and one peak containing the maltose fraction.

The maltose peak is easily identified by comparison to the elution profile of a maltose standard sample. Estimation of the mean molecular weight of the modified starch derivative fraction is performed by comparison to the elution profile, i.e. the retention time, of standard samples having a well-defined mean molecular weight.

The following GPC-conditions are employed:

Column:	Waters Ultrahydrogel 500 10 μM, 7.8*300 mm GPC Column + Waters Ultrahydrogel Guard Column
Eluent:	0.05 M Ammonium formiate, pH 3.5
Column temp.:	Ambient
Injection volume:	10-20 μl
Detection:	Refractive Index Detector
Sample preparation:	Diluted to 1-2% dry matter, filtered using 0.2 μm filters
Standards:	Dextran standards T500, T70 and T10, obtained from Pharmacia Biotech, Sweden.

The present invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Construction of a variant of the maltogenic amylase having the amino acid sequence shown in SEQ ID NO:1 with altered pH dependent activity

The maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 is expressed in *Bacillus subtilis* from a plasmid denoted herein as pLBei010. This plasmid contains *amyM* in which the expression of *amyM* is directed by its own promoter and the complete gene encoding the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1, e.g., as contained in the strain DSM 11837. The plasmid contains the origin of replication, *ori*, from plasmid pUB110 and an kanamycin resistance marker for selection purposes. pLBei010 is shown in Fig. 1 of WO 99/43794.

Primer sequences

Site directed mutants of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 were constructed by the megaprimer method essentially as described by Kammann et al. (1989). Briefly, a mutagenic oligonucleotide primer is used together in a PCR reaction with a suitable opposite DNA strand end primer to create a preliminary PCR product. This product is then used as a megaprimer together with another opposite DNA strand end primer to create a double-stranded DNA product. The product of the final PCR reaction was routinely used to replace a corresponding DNA fragment in the pLBei010 plasmid by standard cloning procedures. Mutants were transformed directly into *Bacillus subtilis* strain SHa273, a derivative of *Bacillus subtilis* 168 which is apr$^-$, npr$^-$, amyE$^-$, amyR2$^-$ and prepared by methods known in the art.

Oligonucleotide primers used in the construction of described variants are as listed below:

Variant Sequence (5'→ 3')
F188H: SEQ ID NO: 3
F188E: SEQ ID NO: 4
F284E: SEQ ID NO: 5
F284D: SEQ ID NO: 6
F284K: SEQ ID NO: 7
N327D: SEQ ID NO: 8
Variant Sequence (3'→ 5')
T288K: SEQ ID NO: 9
T288R: SEQ ID NO: 10

Aspartate variants of F284, T288 and N327 were obtained using primer A189 (SEQ ID NO: 11) and B649 (SEQ ID NO: 12) as end-primers.

F188-variants F188L, T189Y were obtained using primer A82 (SEQ ID NO: 13) and B346 (SEQ ID NO: 14) as end-primers.

PCR products with the desired modification(s) were purified, digested with appropriate enzymes, separated by agarose gel electrophoresis and extracted, ethanol precipitated in the presence of glycogen, resuspended in H_2O, ligated to pLBei010 which had been digested with the same appropriate enzymes, and transformed into *Bacillus subtilis* SHa273. Transformants were checked for size by colony PCR and for the insertion or removal of specific restriction sites by restriction enzyme digestion. Positive colonies were verified by DNA sequencing methods as described in the art.

Fermentation

The *B. subtilis* SHa273 mutant clones were grown overnight on LB-Kana (10 μg/ml)-Starch plates at 37°C. The colonies from the plate were resuspended in 10 ml Luria broth. One-sixth of each of the suspensions were inoculated into a 500 ml shake flasks containing 100 ml PS-1 media, a soy meal/sucrose-based media, kanamycin for a final concentration of 10 μg/ml and 100 μl 5M NaOH. The pH was adjusted to 7.5 with NaOH before inoculation. The cultures were incubated for five days at 30°C with shaking at 270-300 rpm.

Enzyme Purification

Large particles from the media were removed by flocculation before affinity chromatography. Superfloc C521 (American Cyanmide Company) was used as the cationic flocculant and Superfloc A130 (American Cyanmide Company) as the anionic flocculant.

The culture suspension was diluted 1:1 with deionized water and the pH was adjusted to approx. 7.5. A volume of 0.01 ml of 50$^{w}/_{w}$% $CaCl_2$ per ml diluted culture was added during stirring. A volume of 0.015 ml of 20$^{w}/_{w}$% Na-aluminate per ml diluted culture was titrated with 20% formic acid, while keeping the pH between 7 and 8. While stirring 0.025 ml 10$^{v}/_{v}$% of C521 per ml diluted culture was added, followed by 0.05 ml 1$^{w}/_{v}$% A130 per ml diluted culture, or until flocculation was observed. The solution was centrifuged at 4500 rpm for 30 minutes. Filtration was performed using a filter of pore size of 0.45 μm

to exclude larger particles and any remaining bacteria. The filtered solution was stored at -20°C.

Immobilization of α-cyclodextrin to DSV-agarose

One hundred mg of α-cylcodextrin of molecular weight 972.86g/mol (Fluka 28705) was dissolved in 20 ml coupling buffer (0.5M Na_2CO_3, pH 11). Ten ml of DSV-agarose (Mini-Leak, Medium 10-20 mmol/l of divinyl sulfone activated agarose (Kem-En-Tec) was washed thoroughly with deionized water, then dried by suction and transferred to the a-cyclodextrin solution. After the mixture had stirred for 24 hr at ambient temperature, the gel was washed with deionized water, followed by 0.5M $KHCO_3$. The gel was transferred to the blocking buffer (20ml 0.5M $KHCO_3$ + 1ml mercaptoethanol), stirred for 2 hr at ambient temperature, then washed with deionized water.

Affinity chromatography

The variants were purified by affinity chromatography using the Pharmacia FPLC System. A 0.04 volume of 1M Na-acetate pH 5 was added to the filtrate obtained by flocculation to adjust pH and $CaCl_2$ was added to a final concentration of 10^{-10} M. The solution was filtered and degassed. A Pharmacia XK16 column was prepared with ten ml of the immobilised α-cyclodextrin, then equilibrated in the equilibration buffer (25 mM Na-acetate pH 5) by washing with approximately 10 times the column volume. The filtrate was applied to the XK16 column, which was then washed with the equilibration buffer until protein could no longer be detected in the washing buffer. The column was washed with the equilibration buffer containing 0.5M NaCl to elute nonspecific material, followed by another wash with 2-3 times the column volume of the equilibration buffer. All washings were performed using a flow rate of 10ml/min. Specifically bound material was eluted using a solution of 2% α-cyclodextrin in the wash buffer and collected using the Pharmacia Liquid Chromatography Collector LCC-500 Plus using a flow rate of 5 ml/min.

Example 2: pH dependent activity of variants

The variants prepared in the preceding Example were tested for activity at various pH values as follows.

A colorimetric glucose oxidase-peroxidase assay for liberated glucose from maltotriose or amylopectin was used to determine the pH activity profiles of the enzyme variants (Glucose/GOD-Perid® Method, Boehringer Mannheim, Indianapolis IN). Activity was assayed in a buffer of 25 mM citrate-phosphate, 0.1mM $CaCl_2$ at pH values of 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.6. The buffer pH was adjusted using NaOH and

enzymes were diluted in 25 mM citrate-phosphate buffer pH 5. Measurements were taken in duplicate to obtain an average value. All values are relative to the pH at which the highest level of activity is seen.

The results, shown in the table below, indicate that each of the variants has an alteration in the pH dependent activity profile when compared to the parent maltogenic amylase having the amino acid sequence having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1. The highest level of activity for each variant is designated 100% and the activity of that variant measured at the other indicated pH values is a relative percentage of that maximum.

Modifications	pH													
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.6
None (parent)	0	0	0	8	47	80	100	95	91	80	66	39	35	30
F188H	1	0	0	1	3	29	77	99	100	88	59	39	31	27
F188E	0	0	0	2	27	62	89	100	93	71	46	28	20	18
T288R	0	0	0	8	51	77	94	100	86	73	50	34	27	12
N327D	1	1	7	27	67	95	100	98	77	33	19	11	5	0

Further, a number of variants were tested for activity at pH 4.0 and 5.0, taking the activity of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 at the same pH as 100 %. The activity was determined by hydrolysis of maltotriose (10 mg/ml) at 60°C, 50 mM sodium acetate, 1 mM $CaCl_2$. The results are expressed as the ratio between activity at pH 5.0 and pH 4.0:

Modifications	pH 5.0/pH 4.0
N131D	0.24
I174Q	0.31
G397P	0.40
H103Y	0.40
Δ 262-266	0.47
T142A + D261G +T288P +Q449R	0.50

S32Q	0.53
S32D	0.55
T142A+ D261G	0.62
G370N+ N371G	0.66
S32N	0.68
N176S	0.79
D17E	0.80
None (parent)	1
Δ 191	1.39
192-A-193	1.61
I174E	1.80
192-A-G-193	1.90
Δ 192	2.22
F188L + D261G + T288P	2.47

The results demonstrate that variants with a higher or lower pH optimum can indeed be obtained.

Example 3: Thermostability of variants

Incubation at 80°C

The thermostability of a number of variants was tested by incubating an aqueous solution at 80°C, pH 4.3, 50 mM acetate buffer, 1mM $CaCl_2$, and measuring the residual amylase activity at various times. The parent enzyme, the maltogenic amylse having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1, was included for comparison. The results are expressed as residual activity at various times in percent of initial activity:

Variant	0	5 min.	10 min.	15 min.	20 min.	25 min.
None (parent)	100	23	9	3	1	0
A197P + D261G + T288P	100	36	28	14	16	9

+ N342S						
A30D+ K40R+ D261G	100	38	24	15	13	10
T288K	100	64	31	18	7	4
T142A+ N327S+ K425E+ K520R+ N595I	100	47	39	25	19	11
T142A + D261G + T288P + Q449R	100	45	36	27	16	9
K40R+ F188L+ D261G+ A483T	100	56	48	40	36	30
F188L+ V336L+ T525A	100	63	49	48	52	47
F188I+ Y422F+ I660V	100	71	60	51	43	38
N115D+ F188L	100	73	60	51	44	39
F188L+ D261G+ T288P	100	60	67	66	63	67
F188L + D261G + T288P + A483T	100	66	72	73	75	78
N26S + F188L + D261G + T288P + T594A + I600V	100	80	80	82	84	84
N26S + T80A + F188L + D261G + T288P + R291L	100	80	75	82	83	87

The above data show a clearly improved thermostability for the variants compared to the parent amylase. Thus, after 15 minutes incubation at 80°C, a number of variants show at least 25% residual activity, and some even show at least 50% residual activity, whereas the parent enzyme has essentially lost its activity.

Incubation at 85°C

The variant S32E was tested by incubation with 1 mM Ca^{++} at 85°C for 15 minutes. The variant showed a residual activity of 48% whereas the parent enzyme (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1) showed 32% residual activity at the same conditions.

Incubation at 90°C

Four variants and the parent enzyme were tested by incubating at 90°C, pH 5.0, 50 mM acetate buffer, 1 mM $CaCl_2$, and measuring the residual activity. The results were as follows:

Variant	0	10 min.	20 min.	30 min.
None (parent)	100	5	0	0
F188L + D261G + T288P	100	70	41	28
N26S + F188L + D261G + T288P + T594A + I600V	100	71	54	39
N26S + T80A + F188L + D261G + T288P + R291L	100	43	26	13
F188L + D261G + T288P + A483T	100	54	39	26

The variants show a clearly improved thermostability. Thus, the variants retain more than 10 % (or even more than 20 %) relative activity after 30 minutes incubation at 90° C, whereas the parent enzyme loses all activity after 20 minutes.

DSC

Further, the thermostability was tested for some variants by DSC (differential scanning calorimetry) at pH values in the range 4.0-5.5. Again, the parent amylase was included for comparison. The results are expressed as the denaturation temperature (Tm) at the given pH:

Modifications	pH 4.0	pH 4.3	pH 5.0	pH 5.5
None (parent)	64°C	79°C	83°C	88°C
N115D+ F188L		86°C		92°C
T142A+ N327S+ K425E+ K520R+ N595I				93°C
F188L + D261G + T288P	75°C		95°C	

The results show improved thermostability for each variant. One variant shows an improvement of more than 10°C at pH 4.0 and 5.5.

Example 4: Specific activity of variants

Amylase activity was determined by a colorimetric measurement after action on Phadebas tablets at pH 5.0 and 60°C. The results for two variants, relative to the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 were as follows:

Modifications	Relative amylase activity
None (parent)	100
192-A-193	110
Δ (191-195)	300

The specific activity was further tested by action on maltotriose at pH 4.0, 60°C by the MANU method described above. The results showed that the variant G370N, N371G has a maltotriose activity of 106 % compared to the parent maltogenic amylase.

Example 5: Inhibition of retrogradation

The efficiency of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 and variants thereof to inhibit retrogradation was determined as follows:

730 mg of 50 % (w/w) amylopectin slurry in 0.1 M sodium acetate, at a selected pH (3.7, 4.3 or 5.5) was mixed with 20 µl of an enzyme sample, and the mixture was incubated in a sealed ampoule for 1 hour at 40°C, followed by incubation at 100 °C for 1 hour in order to gelatinize the samples. The sample was then aged for 7 days at room temperature to allow recrystallization of the amylopectin. A control without enzyme was included.

After aging, DSC was performed on the sample by scanning from 5°C to 95°C at a constant scan rate of 90°C/hour. The area under the first endothermic peak in the thermogram was taken to represent the amount of retrograded amylopectin, and the relative inhibition of retrogradation was taken as the area reduction (in %) relative to the control without enzyme.

In the table below, the efficiency of the enzyme is expressed as the ratio of the relative inhibition of retrogradation to the enzyme dosage (in MANU/ml):

pH	Modifications	MANU/ml	Relative inhibition	Efficiency
3.7	A30D+ K40R+ D261G	0.23	0.38	1.7
3.7	T142A+ N327S+ K425E+ K520R+ N595I	0.07	0.29	4.1
3.7	None (parent)	0.27	0.38	1.4

4.3	**N115D+ F188L**	0.01	0.18	18
4.3	**None (parent)**	0.27	0.43	1.6
5.5	**Δ (191-195)+ F188L+ T189Y**	0.02	0.12	6
5.5	**Δ (191-195)**	0.02	0.14	7
5.5	**Δ (191-195)**	0.05	0.31	6.2
5.5	**N115D+ F188L**	0.01	0.39	39
5.5	**T142A+ D261G**	0.14	0.53	3.8
5.5	**None (parent)**	0.27	0.49	1.8

The results demonstrate that a number of variants are more efficient than the parent amylase to inhibit retrogradation.

Example 6: Substrate specificity of variants

The activity of variants was tested on two different substrates: glucose release from maltotriose and color release from Phadebas colored starch. The parent enzyme (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1) was tested for comparison. The measurements were made at pH 5, and each activity was expressed relative to the parent enzyme. The ratio of activities on the two substrates was found to be as follows:

Variant	**Activity ratio Starch/maltotriose**
Parent enzyme	1.0
F188L, D261G, T288P	3.6
F188L, D261G, T288P, T594A, I600V	5.5
N26S, T80A, F188L, D261G, T288P, R291L	1.9
A197P, D261G, T288P, N342S	1.5
T142A, D261G, T288P, Q449R	2.5
F188L, D261G, T288P, A483T	2.5

It can be seen that the 6 variants have an increased activity on starch relative to maltotriose.

Example 7: Preparation of an enzymatically modified starch derivative comprising a hydrophilic group

10 MANU/g DS variant (with F188L+D261G+T288P substitutions) is added to a 30% w/w cationic starch (Mylbond 141 from Amylum) suspension. The suspension is heated with direct steam (3°C per minute) to 85°C, where after the starch solution obtained is maintained at 85°C for 10 minutes. The modified starch derivative solution obtained is then passed through a jet-cooker where the enzyme is inactivation at 140°C for 45 seconds. The modified starch derivative solution obtained is then diluted with water to about 20% by weight of dry substance and cooled to 50°C. The viscosity is then measured (Brookfiled, Type LVT, 30 rpm) directly upon the preparation of the solution and subsequently after 1,2,4, and 24 hours storage in an oven.

Example 8: Preparation of an enzymatically modified starch derivative comprising a hydrophilic group

10 MANU/g DS variant (with F188L+D261G+T288P substitutions) is added to a 30% w/w Hydroxy propyl starch (C*Film from Cerestar) suspension. The suspension is heated with direct steam (3°C per minute) to 85°C, where after the starch solution obtained is maintained at 85°C for 10 minutes. The modified starch derivative solution obtained then passed through a jet-cooker where the enzyme is inactivation at 140°C for 45 seconds. The modified starch derivative solution obtained is then diluted with water to about 20% by weight of dry substance and cooled to 50°C. The viscosity is then measured (Brookfiled, Type LVT, 30 rpm) directly upon the preparation of the solution and subsequently after 1,2,4, and 24 hours storage in an oven.

Example 9: Surface-sizing of Paper

Enzymatically modified starch derivative solutions prepared in accordance with Example 7 and 8 are used as surface-sizing agent for paper. The modified starch derivative solutions are applied to a base paper in the form of an aqueous solution by means of a horizontal size press (type T.H. Dixon; model 160-B; roll hardness 80 shore). The surface-sized paper is then dried with an air foil drier to a moisture content of 5% by weight.

Example 10: Production of an enzymatically modified starch derivative

A 30% DS octenyl-succinate starch (OS-starch) suspension was prepared and pH adjusted to 6.0. Enzyme was added in dosages of 1.25 – 2.00 – 2-50 MANU/g DS and the suspension incubated directly at 80, 85 or 90°C with shaking. Enzymes used were the variant F188L+D261G+T288P and the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 (only at 80°C). After incubation for approximately 24h the enzymes were inactivated at 125°C for 15 min and the samples analysed by GPC. Results are shown in the below table giving the percentages of the major peaks and the approximate MW of these. MW's were estimated by comparison to Dextran standards with MW's of 10,000, 70,000 and 500,000, resulting in very rough estimates.

Enzyme	Dosage MANU/g DS	Temp. °C	Peak 1 MW~sub-strate	Peak 2a >500.000	Peak 2b ~300-500.000	Peak 3 <10.000
	1.25	80	5	-	49	46
Variant	2.00	80	1	-	46	53
	2.50	80	0	-	45	55
	1.25	80	20	33	-	47
Parent	2.00	80	15	34	-	51
	2.50	80	12	35	-	53
	1.25	85	6	-	51	43
Variant	2.00	85	0	-	49	51
	2.50	85	0	-	46	54
	1.25	90	17	-	51	32
Variant	2.00	90	7	-	53	40

	2.50	90	4	-	53	43

Area percentages of the major peaks in the chromatogram obtained by GPC. Numbers given with the peak number are approximate MW of the specific peaks.

As seen in the table the wildtype enzyme, i.e. the maltogenic amylase having the amino acid sequence shown s amino acids 1-686 of SEQ ID NO:1, did not perform as good as the variant at 80°C, showing around 15% non-hydrolysed substrate (peak 1). Also the limit dextrin produced by the wild-type enzyme (peak 2a) had a higher MW and a much more uneven MW distribution compared to that of the variant enzyme (peak 2b). For the variant used in this example, working around 85°C seemed to be optimal since all substrate was hydrolysed at these conditions and the percentages of the limit dextrin (peak 2b) slightly higher than at 80°C.

Example 11: Analysis of the retrogradation of enzymatically modified starch derivatives during storage

The enzyme modified starch derivative prepared as described in Example 10 is spray-dried and an aqueous dispersion at 50% DS made. The dispersion is then stored cold at 4-8°C. Periodically samples (including the freshly prepared sample) are evaluated by DSC analysis giving the extent of retrogradation/recrystallization as a function of time. For comparison, the non-enzyme modified OS-starch can be included as well as other samples of emulsifying starches.

Example 12: Stability of emulsions using enzymatically modified starch derivatives.

A 10% DS aqueous dispersion of the dried enzyme modified starch derivative prepared as described in Example 10 is made and agitated slowly. A citrus oil blend is then added to a final concentration of 30% w/w and the mixture emulsified during high speed agitation. The resulting emulsions are then distributed into parallel glass jars and placed at either room temperature or cold storage. The stability of the samples is evaluated by visual examination, judging the separation, oiling and gelling of the emulsions. For comparison, the non-enzyme modified OS-starch can be included as well as other samples of emulsifying starches.

CLAIMS

1. A method for preparing an enzymatically modified starch derivative, the method comprising the following steps:

I) treating a starch derivative with the maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO:1 or a variant thereof, where the variant

a) has maltogenic amylase activity;

b) has at least 70% identity to the amino acids 1-686 shown in SEQ ID NO:1, and

c) has optimum maltogenic amylase activity in the pH range 3.5-7.0; and

II) optionally recovering the enzymatically modified starch derivative.

2. A method for preparing an enzymatically modified starch derivative, the method comprising the following steps:

I) treating starch with the maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO:1 or a variant thereof, where the variant

a) has maltogenic amylase activity,

b) has at least 70% identity to the amino acids 1-686 shown in SEQ ID NO: 1, and

c) has optimum maltogenic amylase activity in the pH range 3.5-7.0;

Ia) chemically deriving the enzymatically modified starch; and

II) optionally recovering the enzymatically modified starch derivative.

3. The method according to claims 1 or 2, wherein step I) is carried out at a temperature above 65°C.

4. The method according to claim 3, wherein step I) is carried out at a temperature about or above 70°C, preferably about or above 75°C, more preferably about or above 80°C, even more preferably about or above 85°C, most preferably about or above 90°C, in particular about or above 95°C.

5. The method according to any of the preceding claims, wherein the modified starch derivative has a mean molecular weight in the range of from 75,000 to 750,000, preferably in the range of from 100,000 to 600,000, such as in the range of from 100,000 to 500,000, when determined by the GPC-method described herein.

6. The method according to any of the preceding claims, wherein the starch has been chemically derived in such a way that the modified starch derivative contains a hydrophobic group or both a hydrophilic group and a hydrophobic group.

7. The method according to claim 6, wherein the hydrophobic group comprises at least 5 carbon atoms, preferably from 5 to 24 carbon atoms.

8. The method according to claim 7, wherein the hydrophobic group is selected from the group consisting of fatty acid, alkyl, alkenyl, aralkyl and aralkenyl.

9. The method according to any of the preceding claims, wherein the modified starch derivative is an octenyl succinate derivative.

10. The method according to any of claims 1-5, wherein the starch has been chemically derived in such a way that the modified starch derivative contains a hydrophilic group.

11. The method according to any of the preceding claims, herein the variant has a residual maltogenic amylase activity of at least 25%, preferably of at least 30%, e.g. of at least 40%, more preferably of at least 50%, such as of at least 60%, even more preferably of at least 70%, e.g. of at least 80%, most preferably of at least 90% after incubation with 1 mM Ca^{++} at pH 4.3, at 80°C for 15 minutes.

12. The method according to any of the preceding claims, wherein the variant has at least 80% identity to the amino acids 1-686 shown in SEQ ID NO:1, preferably at least 90% identity, such as at least 92% identity, more preferably at least 94% identity, such as at least 95% identity, even more preferably at least 96% identity, such as at least 97% identity, e.g. at least 98% identity or at least 99% identity.

13. The method according to any of the preceding claims, wherein the variant has optimum maltogenic activity in the pH range 4-5.5.

14. The method according to any of claims 1-10, wherein the maltogenic amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1.

15. Use of a maltogenic amylase having the amino acids 1-686 shown in SEQ ID NO: 1 or a variant thereof, where the variant

a) has maltogenic amylase activity,

b) has at least 70% identity to the amino acids 1-686 shown in SEQ ID NO: 1, and

c) has optimum maltogenic amylase activity in the range pH 3.5-7.0;

for the preparation of an enzymatically modified starch derivative.

16. Use according to claim 15, wherein the preparation of the modified starch derivative is carried out in accordance with any of claims 1-14.

17. An enzymatically modified starch derivative obtainable by the method defined in any of claims 1-14.

18. An emulsion comprising the modified starch derivative defined in claim 17.

19. A food product comprising the emulsion defined in claim 18.

20. The food product according to claim 19, where the food product is a beverage.

21. A beverage flavour concentrate comprising an emulsion as defined in claim 18.

22. A beverage flavour concentrate according to claim 21, further comprising flavouring oil(s), sweetener(s) and water.

23. A flavouring agent comprising an emulsion as defined in claim 18.

24. Use of an enzymatically starch derivative as defined in claim 17 for the preparation of an emulsion as defined in claim 18, or for the preparation for a food product as defined in claims 19 or 20, or for the preparation of the beverage flavour concentrate defined in claims 21 or 22, or for the preparation of the flavouring agent defined in claim 23.

25. A paper product, wherein the paper product has been treated, preferably coated or sized, with the enzymatically modified starch derivative obtainable by the method defined in claim 10.

26. A paper product comprising the enzymatically modified starch derivative obtainable by the method defined in claim 10.

27. Use of an enzymatically modified starch derivative obtainable by the method defined in claim 10 for coating or sizing paper product.

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(54) Title: MALTOGENIC AMYLASE-MODIFIED STARCH DERIVATIVES

(57) Abstract: The present invention relates to methods for preparing enzymatically modified starch derivatives useful for, e.g., incorporation in emulsions. Suitable enzymes include the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 as well as variants thereof. The present invention also relates to enzymatically modified starch derivatives per se as well as use of modified starch derivatives for preparing various food products.

Attorney Docket No.: **6001.204-US** PATENT

COMBINED DECLARATION FOR UTILITY PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MALTOGENIC AMYLASE-MODIFIED STARCH DERIVATIVES
(Title of the Invention)

the specification of which
[ ] is attached hereto
OR
[X] was filed on (08/21/2000) as PCT International Application Number PCT/DK00/00460.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by an amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international applications(s) for patent or inventor's certificate or of any PCT international applications(s) which designated at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate, or any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Claimed
PA 1999 01219	DK	09/01/1999	[X] YES [ ] NO
			[ ] YES [ ] NO
			[ ] YES [ ] NO
			[ ] YES [ ] NO

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date
60/151847	09/01/1999

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Ser Tyr Gly Leu Tyr Asp Pro Thr Lys Ser Lys Trp Lys Met Tyr Trp
 65                  70                  75                  80

Gly Gly Asp Leu Glu Gly Val Arg Gln Lys Leu Pro Tyr Leu Lys Gln
                 85                  90                  95

Leu Gly Val Thr Thr Ile Trp Leu Ser Pro Val Leu Asp Asn Leu Asp
            100                 105                 110

Thr Leu Ala Gly Thr Asp Asn Thr Gly Tyr His Gly Tyr Trp Thr Arg
        115                 120                 125
```

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Asp Phe Lys Gln Ile Glu Glu His Phe Gly Asn Trp Thr Thr Phe Asp
    130                 135                 140

Thr Leu Val Asn Asp Ala His Gln Asn Gly Ile Lys Val Ile Val Asp
145                 150                 155                 160

Phe Val Pro Asn His Ser Thr Pro Phe Lys Ala Asn Asp Ser Thr Phe
                165                 170                 175

Ala Glu Gly Gly Ala Leu Tyr Asn Asn Gly Thr Tyr Met Gly Asn Tyr
            180                 185                 190

Phe Asp Asp Ala Thr Lys Gly Tyr Phe His His Asn Gly Asp Ile Ser
        195                 200                 205

Asn Trp Asp Asp Arg Tyr Glu Ala Gln Trp Lys Asn Phe Thr Asp Pro
    210                 215                 220

Ala Gly Phe Ser Leu Ala Asp Leu Ser Gln Glu Asn Gly Thr Ile Ala
225                 230                 235                 240

Gln Tyr Leu Thr Asp Ala Ala Val Gln Leu Val Ala His Gly Ala Asp
                245                 250                 255

Gly Leu Arg Ile Asp Ala Val Lys His Phe Asn Ser Gly Phe Ser Lys
            260                 265                 270

Ser Leu Ala Asp Lys Leu Tyr Gln Lys Lys Asp Ile Phe Leu Val Gly
        275                 280                 285

Glu Trp Tyr Gly Asp Asp Pro Gly Thr Ala Asn His Leu Glu Lys Val
    290                 295                 300

Arg Tyr Ala Asn Asn Ser Gly Val Asn Val Leu Asp Phe Asp Leu Asn
305                 310                 315                 320

Thr Val Ile Arg Asn Val Phe Gly Thr Phe Thr Gln Thr Met Tyr Asp
                325                 330                 335

Leu Asn Asn Met Val Asn Gln Thr Gly Asn Glu Tyr Lys Tyr Lys Glu
            340                 345                 350

Asn Leu Ile Thr Phe Ile Asp Asn His Asp Met Ser Arg Phe Leu Ser
        355                 360                 365

Val Asn Ser Asn Lys Ala Asn Leu His Gln Ala Leu Ala Phe Ile Leu
    370                 375                 380
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Thr Ser Arg Gly Thr Pro Ser Ile Tyr Tyr Gly Thr Glu Gln Tyr Met
385                 390                 395                 400

Ala Gly Gly Asn Asp Pro Tyr Asn Arg Gly Met Met Pro Ala Phe Asp
                405                 410                 415

Thr Thr Thr Thr Ala Phe Lys Glu Val Ser Thr Leu Ala Gly Leu Arg
            420                 425                 430

Arg Asn Asn Ala Ala Ile Gln Tyr Gly Thr Thr Thr Gln Arg Trp Ile
        435                 440                 445

Asn Asn Asp Val Tyr Ile Tyr Glu Arg Lys Phe Phe Asn Asp Val Val
    450                 455                 460

Leu Val Ala Ile Asn Arg Asn Thr Gln Ser Ser Tyr Ser Ile Ser Gly
465                 470                 475                 480

Leu Gln Thr Ala Leu Pro Asn Gly Ser Tyr Ala Asp Tyr Leu Ser Gly
                485                 490                 495

Leu Leu Gly Gly Asn Gly Ile Ser Val Ser Asn Gly Ser Val Ala Ser
            500                 505                 510

Phe Thr Leu Ala Pro Gly Ala Val Ser Val Trp Gln Tyr Ser Thr Ser
        515                 520                 525

Ala Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile Pro
    530                 535                 540

Gly Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln Gly
545                 550                 555                 560

Thr Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr Ser
                565                 570                 575

Asn Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr Asp
            580                 585                 590

Val Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr Asn
        595                 600                 605

Ile Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala
    610                 615                 620

Pro Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro
625                 630                 635                 640
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Glu Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala
645 650 655

Gln Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe
660 665 670

Ser Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg
675 680 685

Ala Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr
690 695 700

Thr Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn
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